Role of tricalcium phosphate implant in bridging the large osteoperiosteal
gaps in rabbits

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Treatment options for large osteoperiosteal defects are limited and that which are available are not ideal. Osteoperiosteal
defect were created in ulnae of both forelimbs of rabbits and tricalcium phosphate implant was used to bridge the gap. Amongst
the 35 implanted ulnae, one implant got dislodged. Rest of the implants showed good adherence to host bone until the final
follow up. Five control rabbit limbs (in which no implants were put) showed persistent bone gap. Histological and Electron
microscopic examination revealed bone tissues covering the surface of the implant and bridging the gap. New bone was formed
in the pores also. Tricalcium phosphate implants showed new bone formation due to osteoconductive properties. They are
biodegradable. It is suggested that tricalcium phosphate implants are viable treatment alternatives in management of large
osteoperiosteal defects with minimal to no adverse effects.

Keywords: Ceramic, Osteoconduction, Osteoperiosteal gap, Tricalcium phosphate

Reconstruction of segmental defects of long bones has
been a long standing problem with orthopaedic
surgeons for which an appropriate answer is yet to be
found. Alternatives to autologous bone grafts have
been explored, including allografts of cortical bone
and cortical spacers, such as demineralized bone
matrix products, synthetic bone substitutes etc\textsuperscript{1,2}.
Amongst the various synthetic bone options available
currently, one is the use of bioactive ceramics like
tricalcium phosphate. It has been frequently used in
clinical and pre-clinical trials as filling or coating
material in reconstructive surgery\textsuperscript{3}. Biological,
histological and biomechanical studies regarding
implantation of tricalcium phosphate have revealed
their potent osteoconductive properties and safety\textsuperscript{4}.
There are various studies which show tricalcium
phosphate implants to be effective in healing of
bone gaps but the defect bridged in these studies are
3-4 mm drill holes which may heal spontaneously\textsuperscript{4,5}.
The aim of the present study is to evaluate the role
of tricalcium phosphate implants in bridging large
osteoperiosteal gaps in rabbit ulnae with the help of
clinical, radiological, histological and surface
electron microscopic observations.

Materials and Methods

The implant—The implant was prepared from
synthetic tricalcium phosphate powder (Fig. 1). A slip
was prepared by mixing 40 g tricalcium phosphate
powder with 100 mL distilled water and 4 g of
polyvinyl alcohol (Binder). To prepare a slip cast 100
ml of distilled water was taken and 4 g of polyvinyl
alcohol was added to it and the mixture was heated to
80 to 90 °C till no vapours were seen and uniform
dissolution of polyvinyl alcohol in water occurred.
Then 40 g of tricalcium phosphate was added to it and
the mixture stirred and this was set to cool for next
2-3 h. The prepared slip was poured into plaster of
Paris mold. The mold was kept at room temperature
for 48 to 72 h. Removal of mould produced a solid
cast. These slip casts were then sintered at 1250-1350 °C
for different soaking period ranging from 100 to 400
min dried to produce dense bioactive tricalcium
phosphate with pore size less than 50 µm.

Fig. 1—Cylindrical tricalcium phosphate implant used in the study.
The porosity, pore size and bulk density depends on the heat treatment and soaking periods in the furnace. Care was taken to ensure that porosity did not exceed 40% as porosity adversely affected the strength. The size of the implants ranged between 1.5 and 2 cm in length and 2 and 3 mm in diameter. All implants were cylindrical in shape (Fig.1).

New Zealand white rabbit (*Oryctolagus cuniculus*), was chosen as model due to its easy availability, docile nature and easy maintenance. All animals were maintained as per norms laid down by Committee for Control and Supervision of Experimental Animals at the Central Animal House, Banaras Hindu University (Reg No 542/02/ab CPCSEA) and approval of the Ethical Committee of the Institute was taken. The model was ulna of the rabbit which has been extensively used as it does not need internal fixation and intact radius provides splintage.

**Surgical procedure**—Healthy mature (6-8 weeks old) rabbits (20), irrespective of sex, weighing approx 1.5 kg were selected while 15 rabbits would have both their forelimbs operated and implanted (experimental), 5 rabbits would have both their forelimbs operated but only one limb implanted (control rabbits). Prior to surgery fore-limbs were shaved and cleaned thoroughly with savlon and spirit and painted with betadine lotion.

Under general anaesthesia using intraperitoneal sodium pentobarbitone (7 mg/kg), rabbits were operated in lateral position. About 4 cm long incision was given on posterior subcutaneous border of ulna in middle 1/3. After incising skin, fascia and muscle to reach the bone, careful extraperiosteal dissection was done and an osteoperiosteal gap of 1.5-2.0 cm was created. An autoclaved implant (about 1-2 mm longer than the gap surgically created) was inserted in the gap. Fitting of the implant was observed at both proximal and distal ends. Post operatively plaster cast support was given for 2 weeks.

**Post-operative schedule**—Animals were kept in separate cages at of 25 °C and RH 55% and fed on a standard rabbit feed and water. One 25 mg dose of tetracycline was given preoperatively and one dose postoperatively intramuscularly. Incision wounds were examined daily for 7 days for any infection, swelling gaping of wound or skin necrosis. The rabbits were followed up for 12 to 36 weeks.

For this study four modalities of investigation were used: Roentgenograms, gross examination, histopathology and scanning electron microscopy.

**Roentgenograms**—Serial roentgenograms at 4 week intervals were taken and following were observed:

1. Position of the implant.
2. New bone formation at proximal and distal host implant junction and bonding.
3. The density of implant.
4. Any fracture of the implant or bone.
5. Density of the new bone and remodeling.
6. If a gap between host-implant junction existed initially whether it diminished or remained as such.

**Macroscopic examination**—At different intervals rabbits were sacrificed and forearm bones were dissected out and examined for mobility, position and consistency of the implant and any new bone formation at proximal or distal host implant junction.

**Histopathology**—The specimens cut from the implant and the host implant junctions for histological examination were decalcified by Gooding and Steward reagent. They were fixed in 10% neutral formalin and dehydrated in graded ethanol (30-100%). Sections of 5-8 µm thickness were cut by microtome. Sections were stained with haemotoxylin and eosin and mounted with Canada balsam.

**Scanning electron microscopy examination (SEM)**—Samples were fixed in 20% glutaraldehyde for 30 min and washed with 0.1M sodium cacodylate buffer. They were dehydrated in graded concentration of alcohol. Samples were dried and coated with gold palladium in iron coater. Prepared sections were observed under Jeol 840A scanning electron microscope.

**Results**

Of the twenty rabbits used, one died during the operation due to high dose of anesthesia. Of the rest 19 rabbits, 1 rabbit died after 12 weeks (cause of death not known) of operation (R-18). Among 18 rabbits, 4 were sacrificed after 12 weeks, 3 after 16 weeks, 7 after 24 weeks and 4 after 36 weeks. All rabbits were bearing weight in the post-operative period. All wounds healed with primary intention without infection and there was no sign suggestive of immunological reaction such as oedema, loss of hair, discharge or rejection of implant. All animals gained weight normally.

**Roentgengraphic study (Fig.2)**—Five rabbits (R-1,2,3,4 and 5) were used as control. Post operatively roentgenogram showed gaps in all control.
rabbit limbs which persisted till the last radiological follow up and the fracture ends gradually became tapered and sclerosed indicating gap non union (16 weeks-R1, 2, 24 weeks-3, 4, 32 weeks-R5). Among the experimental rabbits implant was in position during the entire follow up of 36 weeks except one (R7). By 8 weeks follow up most cases were showing new bone formation at the bone implant junction and the amount of new bone progressively increase with further follow up. Rabbits (4) that were sacrificed at 36 weeks follow up showed increase radiological lucency of the implant indicating biodegradation of tricalcium phosphate and creeping substitution by new host bone.

Macroscopic examination—The post-mortem specimens were subjected to gross examination. Subcutaneously the implant was palpable and side to side mobility of implant was assessed. By 4 weeks follow up all cases were showing minimal abnormal mobility at the fracture ends indicating beginning of formation implant bone adhesion. By 8 weeks there was no abnormal mobility at the implant bone interface in the experimental group (18 rabbits), indicating good bonding (Fig. 3) between the implant and the host bone (except for one rabbit R7 in which the implant was displaced from the beginning). In the control group all rabbits showed abnormal side to side mobility at the fracture gap throughout the follow up period.

Histopathological examination—Histopathological examination through the bone implant junction at various levels was done (Fig. 4). All the cases showed new bone formation from scanty to well-formed woven bone both peri-implant and through the implant. By 12 weeks the specimens showed new bone formation with fibrosis and collagenisation at junctional area and throughout the implant. Porous amorphous implant material could still be seen in the centre surrounded by irregular peri-implant new bone formation. In this new bone lacunar spaces filled with osteocytes were also seen. At places mesenchymal tissue proliferation was seen. Some new bone was seen within the pores of the implant haphazardly. By 36 weeks follow up the specimens were showing lamellar bone formation at the bone implant junctional area and also at many places within the implant. In between new bone tricalcium phosphate particles were present.

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Fig. 2—Serial follow up radiographs of one of the cases showing radiographs taken (from left to right) at 12, 16, 24 and 36 weeks respectively. Radiographs show good incorporation of implant into the bone at the ends with no gap visible.

Fig. 3—Gross specimen of resected forelimb showing incorporation of the implant into the host bone at either ends.

Fig. 4—Histopathology showing porous amorphous implant material on right side surrounded by irregular peri-implant new bone formation. In this new bone lacunar spaces filled with osteocytes are also seen. Some new bone is seen within the pores of the implant haphazardly (100X).
Scanning electron microscopy (SEM) (Fig. 5—Tricalcium phosphate implant along with bone junctional area about 1 cm long (0.5 cm of each) was taken for surface electron microscopy. The surface of the implant showed bone growth and some porosity. The surface of the bone was seen at 150 X magnification. The crystal structure of bone was clear and junctional area showed good bonding between implant and bone. No gap could be discerned at the host implant interface after 20 weeks in all implanted specimens.

Discussion
There has long been a search for a material to replace lost human bone due to various causes. Autogenous bone, allograft and xenograft all have their own advantages and disadvantages. In the present era great interest has been generated in development and use of synthetic bone substitutes and foremost amongst them are ceramics. The clinical goal when using ceramic biomaterials, as is the case with any biomaterial, is to replace lost tissue or organ structure and/or function. The rationale for using ceramics in medicine and dentistry was initially based on the relative biological inertness of ceramic materials compared with metals. However, in the past two decades, this emphasis has shifted more toward the use of bioactive ceramics, materials that not only elicit normal tissue formation but may also form an intimate bond with bone tissue. The differences in structure, composition and physical characteristics of the synthetic tricalcium phosphate arise as a result of processing. Important physical properties are the specific surface area, the particle shape and size, the crystal structure and crystal size, the residual porosity after sintering, the pore size and shape and the pore size distribution.

Ceramic biomaterials are processed to yield one of four general types of surfaces and associated mechanisms of tissue attachment:

1. Fully dense, relatively inert crystalline ceramics that attach to tissue by either a press fit, tissue ongrowth onto a roughened surface, or a grouting agent;
2. Porous, relatively inert ceramics into which tissue ingrowth occurs, creating a mechanical attachment;
3. Fully dense, surface-active ceramics that attach to tissue via a chemical bond; and
4. Resorbable ceramics that integrate with tissue and eventually are replaced by host tissue.

In the present study dense bioactive ceramic was used as implant material to provide for the structural support needed to withstand the loads across a large osteoperiosteal gap. Calcium phosphate ceramics due to their similarity to mineral portion of bone tissue, relative ease in processing and good cell attachment have been used as bone substitutes, coatings, cements, drug delivery systems and tissue engineering scaffolds. Various advantages it has over bone autografts and allografts include its biocompatibility, safety, predictability, unlimited availability, lower morbidity for the patient and cost effectiveness and make them a good choice for reconstructive surgery, orthopaedics, dentistry, maxillo and craniofacial surgeries, spinal arthrodesis and neurosurgery.

The previous experiments done in this field were implantation of various forms of tricalcium phosphate in holes drilled in cortical or cancellous regions. These were about 3 mm in diameter and implanted in distal femur or proximal tibia or skull in rabbits. However such defects were either too small or site were such that they occasionally may have healed spontaneously. In addition they were surrounded by periosteum. Tricalcium phosphate has not been used previously to fill large osteoperiosteal gaps. The 2-3 cm long osteoperiosteal gap in ulnae of rabbits cannot heal spontaneously until the gap is filled with autograft or suitable bone substitute.

Herokazu et al. have evaluated the role of β-tricalcium phosphate (β-TCP) granules, collagen, and fibroblast growth factor-2 (FGF-2) on segmental bone gap of 5 mm in rabbit tibiae. The objective of the study was to evaluate the effects of a complex of
β-TCP granules, collagen, and FGF-2 on cortical bone repair in rabbits. They were of the opinion that resorption of tricalcium phosphate is important for bone formation and addition of FGF-2 improved the strength of the construct. The present study also supports this hypothesis. The tricalcium phosphate implant was poorly resorbed even after 36 weeks of implantation. There were only 3 implants from a total of 19 implanted limbs that showed reduced density of the implant at 24 weeks follow up. Considering the strength of the implant necessary for the big osteoperiosteal gap that was created, dense bioactive ceramic was used in the present study.

The tricalcium phosphate 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 (Fig. 4) 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.

Uchida et al. had reported bone ingrowth in most of the pores in 3 types of ceramics at 12 months when used in rabbit skull and complete bone incorporation was reported by Bucholz et al. and Holmes et al. In contrast to the above studies, in the present study there were large areas of the central part of the tricalcium phosphate implant that were devoid of new bone, although many cases showed bone growth on the surface of the implant as seen by electron microscopy. This shows the inability of dense tricalcium phosphate implant to be degraded completely and to be replaced by normal bone when used in large bone defects. The present results also confirm the earlier observations that biodegradation of tricalcium phosphate implant and replacement by bony tissue occurred from periphery to centre.

Ingrowth of blood vessels should be allowed by an ideal bioactive ceramic to sustain the survival of transplanted cells and for osteoconductivity and it should have sufficient mechanical strength to support loads acting at the implant site. It is particularly difficult to incorporate both these criteria into a single ceramic design as osteoconduction and biodegradation is typically maximized by maximizing porosity, whereas mechanical properties are frequently maximized by minimizing porosity.

Tricalcium phosphate is bioactive, is evidenced by the bonding between native bone and the implant as shown by no side to side mobility between the native bone and the implant in most cases and further no gap was visible even in surface electron microscopy (Fig. 5). The bond between bone and tricalcium phosphate implant at the periphery of the implant showed fibrous connective tissue and osteoid initially. Later specimens showed its conversion to bone which at first was in the form of spicules but later took the characteristics of lamellar bone. Some of the tricalcium phosphate particles were incorporated in between the new bone and there were no cells which showed foreign body reaction. This indicates the biocompatibility and biodegradation of tricalcium phosphate. However since dense form of implant (pore size less than 50 µm) was used, the biodegradation was confined to the periphery only.

Tricalcium phosphate does not have osteogenic properties. This was inferred from the displaced implants in rabbit limbs (rabbit number 7, 8) which did not show any heterotopic bone formation in the soft tissues into which the implants were displaced. It has osteoconductive properties as in the periphery of the implant there was new bone formation to some distance and in few limbs bone was seen to be covering the entire implant surface as seen by electron microscope, though interior was still tricalcium phosphate. The close chemical resemblance of tricalcium phosphate with natural osteoid is a possible explanation of lack of any foreign body reaction towards it. In the present study even at 36 weeks follow up there were no signs of infection or foreign body reaction (early or late) either histologically or clinically. All animals gained weight normally and did not show any systemic adverse effects.

Human studies regarding use of ceramics in large osteoperiosteal defects are few. Marcacci et al. treated 4 patients with diphysseal defect of long bones with culture expanded bone marrow cells seeded on porous hydroxyapatite ceramic scaffold designed to match the defect and stabilized the construct with Ilizarov ring fixator or monoaxial fixator. They showed good long term results with complete fusion of implant with host bone and no episodes of implant fractures. In the present study too there were no cases of late loosening of implant bone junction or implant fractures even at 9 months follow up.

Conclusion

Tricalcium phosphate is a bioactive ceramic with osteoconductive, biodegradable, biocompatible
properties but is not osteogenic. Tricalcium phosphate implants are very useful tools in the armamentarium of orthopaedic and plastic surgeons in management of large osteoperiosteal defects of long bones. They show good osteointegration with the host bone with very minimal chances of late dislodgement. However dense tricalcium phosphate implant has slow rate of resorption especially when fabricated into large implants. Tricalcium phosphate implants may hold the key to future reconstructive surgical practice.

Conflict of interest
No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article. No funds were received in support of this study.

References