DEMINERALIZED BONE PARTICLES

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Demineralized bone matrix has been used as a bone graft alternative successfully owing to its osteoinductive activity. Almost all demineralized bone matrix applied clinically to augment bone repair are allogenic,prepared from human cadavers. This report convered the preparation processes of demineralized bone particles from cattle bone, dog bone, human cadaver and corticocancellous bone remains from orthopaedic procedures. Characterization of demineralized bone particle from cattle bone was studied in terms of phase analysis, protein content, morphology, particle distribution and impurity contamination. Allogenic demineralized bone particles from dog bone and xenogenic, from human bone were experimentally implanted into soft tissue and femoral bones of twenty dogs. Follow up period was up to six months. Clinically and histologically both allogenic and xenogenic demineralized bone particles were biocompatible. Roentgenographic, Scanning electron microscopic, and magnetic resonance image intensifier examinations revealed scanty new bone formation at the implanted sites and area of bone-material interface bonding at six month. Human demineralized bone particles had also undergone clinical trials in a few volunteers with reasonable good response.

INTRODUCTION

Bone grafting is frequently used to augment bone healing with the numerous approaches to reconstructing, replacing skeletal defects., to assisting fracture repair, to strengthening arthrodesis and to filling voids after the treatment of bone tumors. For over 100 years, autologous cancellous bone grafting has been the standard of care. Autologous cancellous bone graft is the most effective grafting material because it provides the three elements required for bone regeneration : osteoconduction, osteoinduction, and osteogenic cells. However, because autogenous grafting is associated with several short comings and complications, including limited quantities of bone for harvest and donor-site morbidity including infections, pain, increased anesthesia time, and significantly increased operative blood loss. Alternatives to autogenous bone graft have been sought in an effort to increase the quantity of bone obtained and decrease the morbidity of the grafting process.

BONE GRAFT SUBSTITUTE

Ideally, graft substitutes should provide four elements : (a) an osteoconductive matrix, which is a nonviable scaffolding conductive to bone ingrowth; (b) osteoinductive factors, which are the chemical agents that induce the various stages of bone regeneration and repair; (c) osteogenic cells, which have the potential to differentiate and facilitate the various stages of bone regeneration, (d) structural integrity.

Currently available graft substitutes that are widely used in clinical situations are allograft, ceramics, demineralized bone matrix, bone marrow, and composite grafts. The following table displays the differences in properties of bone-graft alternatives.

Properties of bone-graft alternatives[2,8,9,10]

Grafting material	Osteo-conduction	Osteo-induction	Osteo-progenitor cells	Immuno genicity	Donor-site Morbidity	Immediate torque strength
Cancellous autologous graft	++++	++	+++	-	+	-
Cortical autologous graft	+	+/-	+/-	-	+	++
Fresh allograft	+	+/-	-	++	-	++
Frozen allograft	+	+/-	-	+	-	++
Ceramics	+	-	-	-	-	+/-
Demineralized bone matrix	+	++	-	-	-	-
Bone marrow	-	+/-	++	-	-	-
Particulate ceramic with bone marrow	++	+/-	++	-	-	-

It is noted that Demineralized bone matrix has osteoconductive and osteoinductive properties. There were many clinical trials of the use of demineralized bone matrix with variable results.

Mulliken et al[6] , in 1981, reported the use of demineralized allogenic bone implants for the correction of maxillocanio facial deformities in 44 patients. By radiographic examination, 3 of 19 were healed by three months and an additional 11 were positive by six months. This allogenic bone was obtained from human cadavers and prepared as powders, chips or blocks, and was demineralized.

Kaban et al[13] , in 1982, reported the results of treatment of 50 jaw defects with demineralized bone implants. The radiographic documentation of bone formation has been disappointing. The earliest evidence of calcification across the defect occurred in six months in the majority of cases. This was in constrast to clinical situation when these same defects become solid to palpation after several weeks. These bone implants were obtained from human cadavers and prepared after demineralization in many forms : powder (75-250 μm particle size) ; cancellous spongelike ships, blocks, and strips ; and corticocancellous blocks.

Sonis et al[7] , in 1983, reported the clinical trial of demineralized bone powder in the treatment of peridontal defects in 21 patients. Radiographic suggestion of osseous healing of defects was noted by four months postoperratively in all 11 of 18 evaluable patients. This demineralized bone powder was prepared from human allogenic femoral bone.

Gepstein et al[1] , in 1987, experimented in 33 rats by bridging large defects in radial bones with demineralized bone matrix in the form of a powder. At 5 weeks, the experimental defect was fully bridged, forming solid bone in 71% of the rats, and the remaining 29% showed bridging of 95.8% of the length of the defect (defect length was more than 50% of total length of the bone). This powder was prepared from the diaphyses of 450 gram Long-Evans rats.

STUDY DESIGNS AND OBJECTIVES

It could be seen that demineralized bone matrix in whatever forms as reported in the literatures was mostly prepared from human cadavers and if from animal, rat. The aim of our study is to report our experience of demineralized bone matrix prepared from different sources.

1. Preparation of demineralized bone particles (DBP) from a) cattle bone b) dog bone and c) cadaveric human cortical bone and d) cancellous bone remains from orthopaedic proceedures.

2. Characterization of bone particles prepared from cattle bone, before and after demineralizaton.

- 2.1 Phase analysis by X-ray Diffractormeter(Phillip)
- 2.2 Protein content study by Macro Kjeldahl (Kjeltec KD-02)
- 2.3 Morphologic study by SEM (Jeol T220A)
- 2.4 Particle size study by particle size analyzer (Malvern, Mastersizer x Ver 1.0)
- 2.5 Impurities contaminated study by Atomic Absorption Spectrometer (Varian, spectr AA 300/400)

3. Experimental studies in dog
   - 3.1 implantation of dog DBP into bone and soft tissue of dogs.
   - 3.2 implantation of human DBP into bone and soft tissue of dogs.
   - 3.3 histological study for biocompatibility.
   - 3.4 radiological study for new bone formation.
   - 3.5 scanning electron microscopic study for material-bone interface.
   - 3.6 magnetic resonance image intensifying study for tissue around DBP.
4. Early clinical trials in human
   - 4.1 implantation of human DBP in bone.
   - 4.2 implantation of human DBP in soft tissue.
   - 4.3 radiological study.
   - 4.4 macro-and microscopic studies.

EXPERIMENTAL STUDY OF DBP MATERIALS

I. Preparation of demineralized bone particles from cattle bone[4,11]

1. Donor bones are cleaned off adherent periosteum, muscle, and conective tissue. Cartilage is removed. Cancellous bone is separated from dense cortical bone and processed as large pieces. The porous quality of concellous bone simultaneously provides structure and mass and sufficient surface area for osteoinduction. The cortical bone is cut into small pieces to improve the efficiency of subsequent washes and extractions. Denser bone from large animals may need to be frozen and hammered in order to produce chips less than 1 cm. The pieces of bone are thoroughly washed free of marrow and soft tissue with cold, deionized water.

2. The cleaned tissue is extracted with frequent changes of absolute ethanol for at least 1 hour (a total of 4 liters per 100 gm of bone).

3. The tissue is extracted with frequent changes of anhydrous ethyl ether in a fume hood for 1 hour (2 liters per 100 gm of bone). The bone is dehydrated by these extractions and can be stored at room temperature.

4. The dehydrated material is frozen and pulverized in a liquid nitrogen impacting mill (Spex, Metuchen, New Jersey).

5. Pulverized bone particles are sieved into fractions of 75 to 250 to 450, and greater than 450 μm.

6. Bone particle fractions are demineralized with 0.5M hydrochloric acid (50 ml per gm) for 3 hours at room temperature or at 4°C on magnetic stirrers with insulation to prevent overheating. Large chips and blocks are extracted completely at 4°C with frequent changes of 0.5 M hydrochloric acid. Completeness of demineralization can be monitored radiographically, by ashing, or by nondecalcified histologic techniques (von Kossa stain).

7. Acid and liberated minerals are washed away with cold, deionized water until the pH of the wash matches the pH of the water. The water washes can be decanted from the large particles and chips, but must be removed by centrifugation from the fine particles. This requires approximately 500 ml of water per gram of starting bone particles.

8. Demineralized bone powders are extracted with changes of anhydrous ethyl ether for 1 hour (100 ml per gm of starting bone particles). After the last change of ether is removed, the demineralized bone powder is left overnight in the hood until all the residual ether has vaporized. The particles should be odorless, white, and discrete. To minimize static electricity, demineralizd bone powder should be stored in glass, not plastic, containers.

9. The material is extracted in fume hood with changes of anhydrous ethyl ether for 1 hour (100 ml per gm of starting bone particles). After the last change of ether is removed, the demineralized bone powder is left overnight in the hood until all the residual ether has vaporized. The particles should be odorless, snow-white, and discrete. To minimize static electricity, demineralized bone powder should be stored in glass, not plastic, containers.

10. Cold ethylene oxide or 2 mRAD cathode ray irradiation may be used for sterilization

II. Analytical results of DBP from cattle bone

1. General appearance

Demineralized bone particles extracted from cattle bone had pale yellowish white colour. The same shade of colour also found in DBP from dog and human bones.

2. Phase analysis

XRD pattern of DBP before demineralization was quite different from XRD pattern of DBP after demineralization (Figure 1&2). This was because hydroxyapatite was washed away by acid during the process.

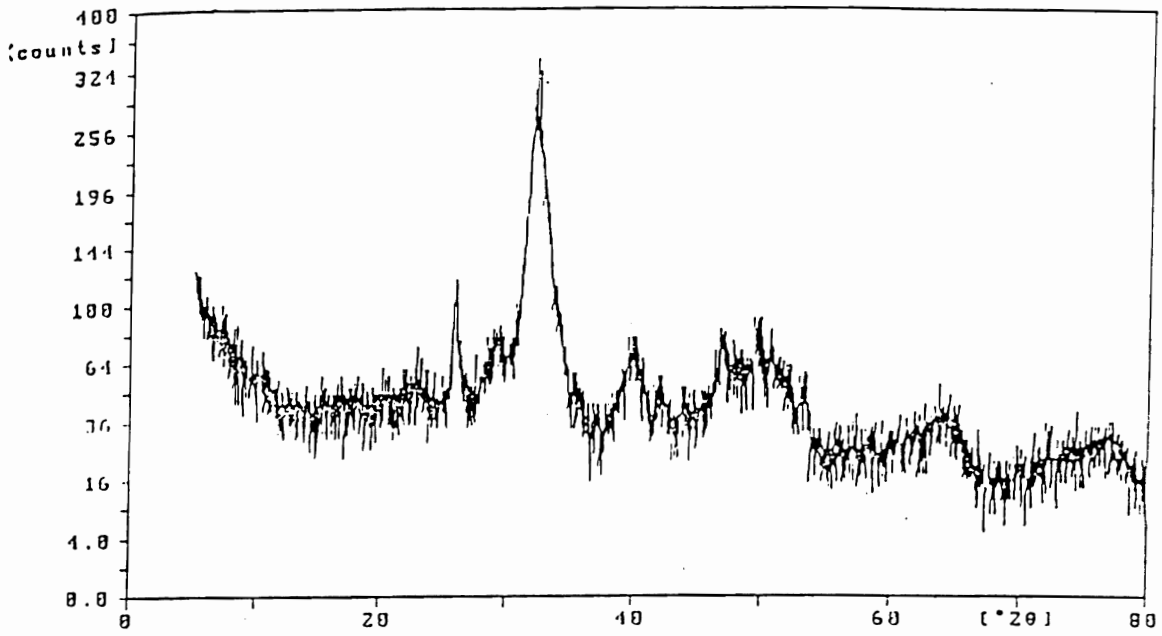

Fig. 1. XRD pattern of non-mineralized bone partides

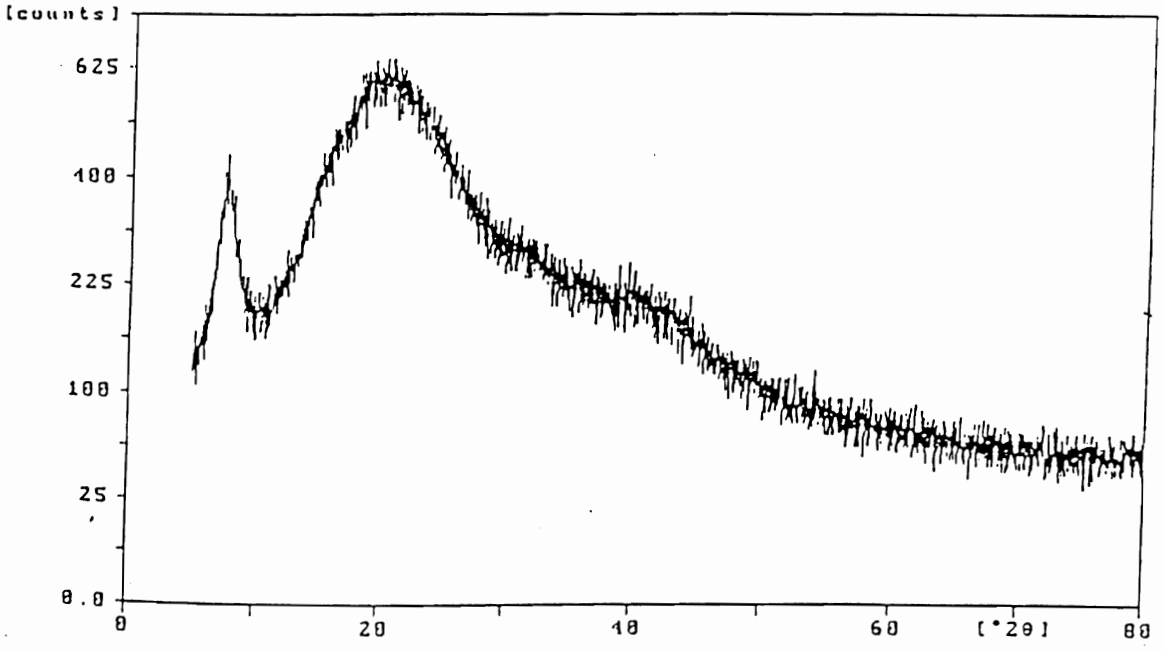

Fig. 2. XRD pattern of demineralized bone particles

In order to confirm this, non-mineralized DBP was calcined at different temperatures as shown in Table 1.

Table 1. Results of calcined non-mineralized DBP

Temperature (°C)	Calcined condition Time (hr.)	Product appearance
400	1	brownish powder
500	1	brownish powder
600	1	brownish powder
700	1	white powder

At 700°C the organic components of non-mineralized DBP was completely burnt away. With XRD examination it was proved that the remain was hydroxyapatite (Fig.3.)

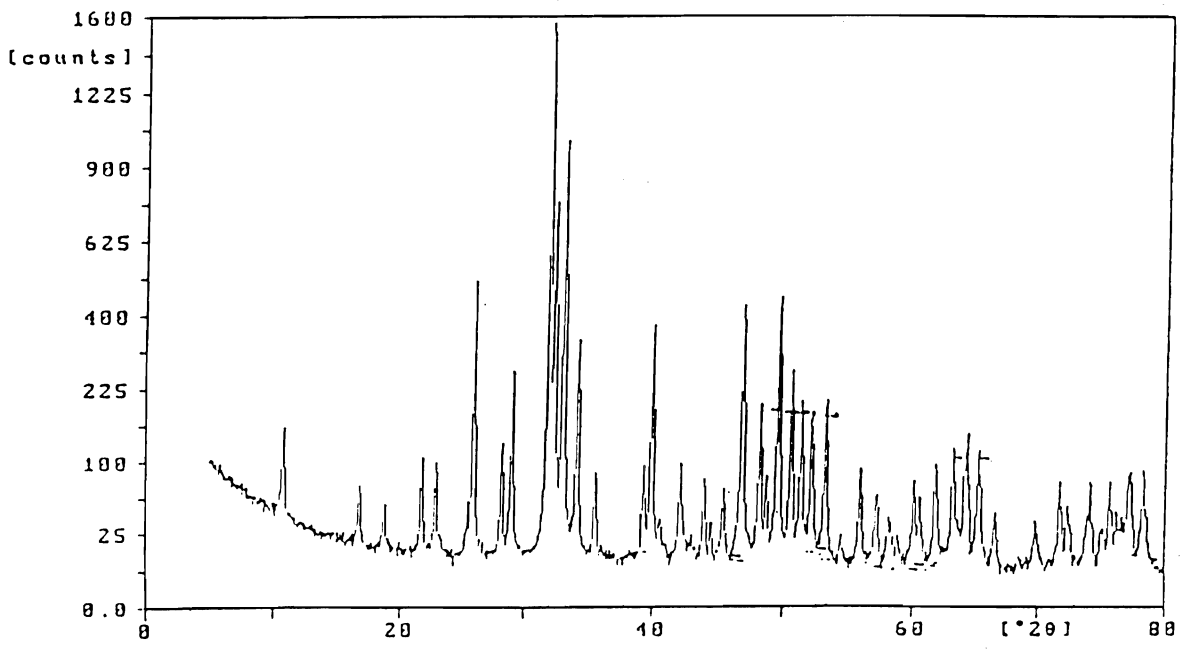

Fig. 3. XRD pattern of calcined non-mineralized DBP at 700°C(1 hr)

Comparison of all three XRD patterns in Fig. 4. showed that XRD pattern of DBP contained no hydroxyapatite peaks.

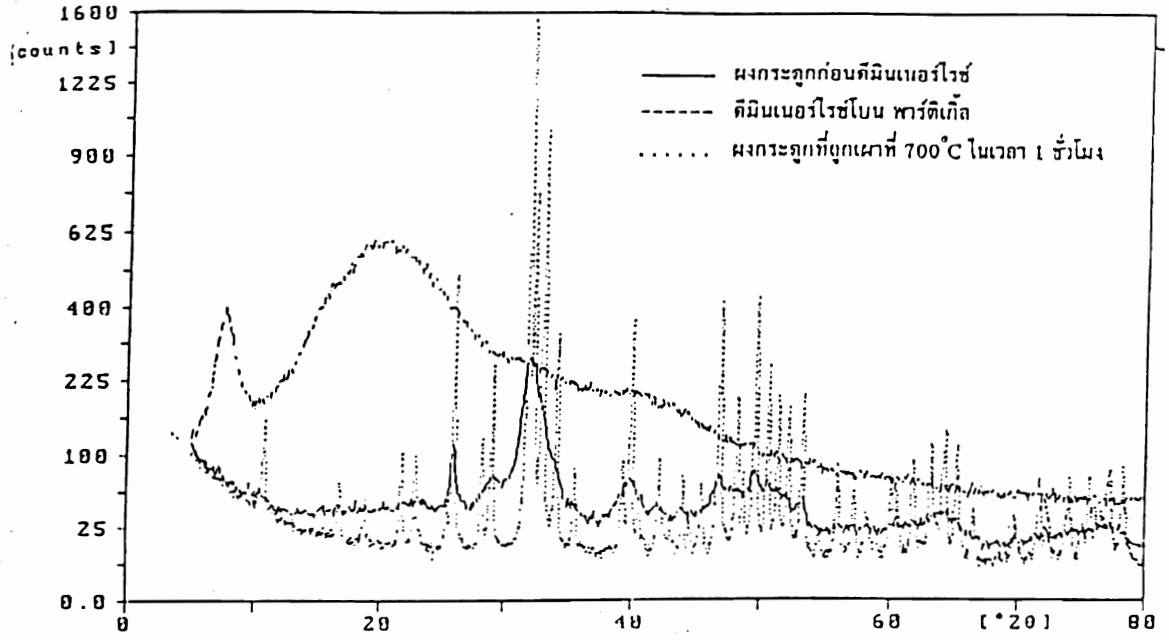

Fig.4. Comparison of three XRD patterns

3. Protein content (Table2)

Sample	Protein (%)
DBP	100%
Befoe demineralized DBP	26.25%

The results showed that no hydroxyapatite was detected in DBP.

4. Morphologic study

From SEM micrographs of 75-425 μm in dimensions of DBP and non-mineralized DBP, it was noted that their shapes were variable, though DBP had more or less equal in size among them than the non-mineralized ones. DBP had also less crack appearance at their surfaces.

Fig 5. SEM micrograph of 75-250 μm (x100) of non-mineralized DBP

Fig 6. SEM micrograph of 75-250 μm (x100) of DBP

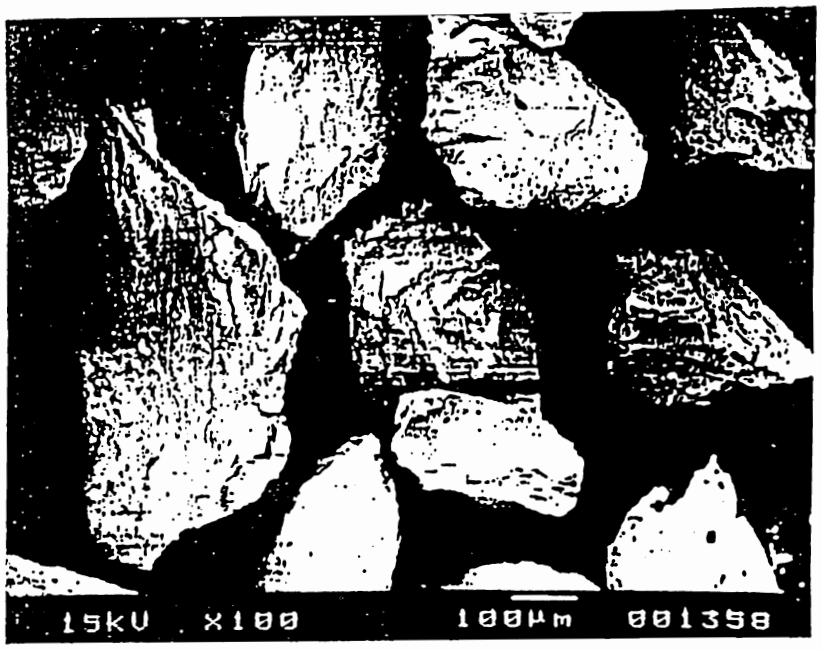

Fig 7. SEM micrograph of 250-425μm (x100) of non-mineralized DBP

Fig 8. SEM micrograph of 250-425 μm (x100) of DBP

5. Particle distribution

Table 2. Distribution of particles of varius sizes

Samples	Particle size (μm)		
	Do.1	**Do.5**	**Do.9**
non-DBP (sieved 75-250 μm)	96.75	205.46	361.10
DBP (sieved 75-250 μm)	107.36	215.43	373.63
non-DBP (sieved 250-425 μm)	941.64	1470.26	1889.53
DBP (sieved 250-425 μm)	860.96	1350.59	1850.29

It was noted from the table above that the particles might have their size larger than the siever. This was due to the spindle-shaped particle.

6. Impurities content

Table 3. Heavy metals in cattle bone

Heavy metals	Quantity (ppm)
As	<0.05
Pb	1.64
Cd	0.07

It could be seen that the amount of heavy metals, As, Pb and Cd was less than 2 ppm. which was considered insignificant to cause any harmful effects when implanting in animal experiment.

Preparation of dog and human DBP were also executed in the same manner as cattle bone. However, characterization of dog and human DBP was not performed, assuming that their characters would be the same as cattle DBP.

EXPERIMENTAL STUDY IN DOG

This was done by implantation of dog DBP and human DBP into the soft tissue, muscle and into the femoral shaft and condyle of twenty dogs. Clinical observation was followed up to six months postoperatively, during which roentgenographs were taken periodically at 1,3 and 6 months. Histological study was done by examining soft tissue and muscle at one month. SEM study and an attempt to study with magnetic resonance image intensifier were also applied.

Clinically all wenty dog lived their normal lives after surgery. There were no complication related to surgical procedures and no signs of inflammation or infection at the operative limbs where either dog DBP or human DBP were implanted into their soft tissue and bone.

Histologically, the sections, taken at one month after dog and human DBP implantation, showed no sign foreign body reaction, though a few slides did reveal some lymphocytes infiltration and scanty presence of Langhan's giant cells. This could be concluded that both dog and human DBP were biocompatible in dog subjects.

In roentgenographic study, even after follow up to six months, there were no signs of complete bone bridging at the defects in dog femoral shafts and condyles, filled with either dog or human DBP. There were evidence of some new bone formation in and around the defects.

Scanning electron microscopic examination of the bone-DBP interface from the specimens taken at six month showing some areas of good bonding of the interface. However, in other areas there were still gaps at the interface. There was no significant difference between the human DBP and dog DBP.

Eventhough computerized tomography would be prefered to magnetic resonance image intensifier in evaluation the presence of new bone formation of the subjects. The MRI study in this situation revealed nothing solid concerning new bone formation of the dogs examined at six months.

CLINICAL TRIALS IN HUMAN

Human DBP was implanted into shaft of humerus, proximal ulna and ilium wings of 5 volunteer patients. The following up period has been six month now. Clinically and histologically there was no sign

of infection, inflammation and rejection at the operative sites. All the wound looked fine and healthy.

Roentgenographic following up studies up to six month of all the patients, there revealed some new bone formation at the operative defects, filled with DBP. The amount of new bone was apparantly present in the trial group more than in those controls.

DISCUSSION

In general , demineralized bone implants stimulate healing within 3 to 6 month[1,2,5,6,7] Howerver, these were not the experience in certain reports[3,7] including this study. We shared the same opinion with Kaban et al, that there were evidence of calcification across the defects at six month, though Kaban used only human DBP and our series, dog and human. In most reported series, though the concrete quality of DBP used to implant was not mentioned, the particle size of 75-250 μm was generally accepted the most appropriate. In this study, we had proved that the DBP was free from hydroxyapatite and had no significant heavy metals. The reason why our cases did show slow induction of new bone was perhaps the amount of DBP implanted. As already known that DBP contains about 5% of bone inductive proteins, osteocalcin, osteonectin and other growth factors. These bone inductive proteins are significant. The more quantity of DBP implanted, the more quantity of bone inductive proteins would be present. In our series, the amount of implanted DBP was rather small, this might explain why calcification did appear late.

Glowacki et al[2] reported a successful case in human subject using demineralized bovine bone, that was xenogenic. Though we had no such study, we did use human DBP in dog bone without any adverse affects. With careful preparation of donor bone, in order to remove all cellular and potentially antigenic components, xenoimplants may provide an unlimited supply of banked material for osseous surgery.

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