

# Graft perforations favor osteoinduction.

## Studies of rabbit cortical grafts sterilized with ethylene oxide

Göran Sigholm<sup>1</sup>, El Gendler<sup>2</sup>, Harry McKellop<sup>3</sup>, G. June Marshall<sup>4</sup>, Tillman M. Moore<sup>2</sup> and Augusto Sarmiento<sup>5</sup>

The healing of freeze-dried, ethylene oxide sterilized, segmental, allogenic cortical bone grafts was investigated in 15 rabbits using a 2-cm ulnar diaphyseal defect. Five different groups of bone grafts were evaluated: 1) unperforated undemineralized, 2) perforated undemineralized, 3) unperforated demineralized, 4) perforated demineralized, and 5) perforated demineralized grafts enclosed by silicone rubber (Silastic) sheets. There were 3 animals in each group. At 18 days, the study was terminated, and the

implants were examined using radiographs and qualitative histologic preparations.

We observed that healing of perforated demineralized bone was superior to unperforated demineralized bone, that undemineralized bone was partially sequestered in reactive lacunae, and that perforations in demineralized bone became centers of osteoinduction. Demineralized bone sterilized with ethylene oxide by this method vigorously formed new bone.

<sup>1</sup>Formerly Department of Orthopedics, East Hospital, Gothenburg, Sweden; <sup>2</sup>Pacific Coast Tissue Bank, Los Angeles; <sup>3</sup>Biomechanics, USC Department of Orthopedics, Los Angeles; <sup>4</sup>Orthopedic Hospital, Los Angeles; and <sup>5</sup>USC Department of Orthopedics, Los Angeles, CA, U.S.A.

Correspondence: Dr. Göran Sigholm, Läkarhuset, Södra vägen 27, S-411 35 Gothenburg, Sweden

Tel +46-31 810900. Fax +46-31 186333

Submitted 90-05-01. Accepted 91-10-31

It has been suggested that demineralized bone as intact segments (Tuli and Singh 1978, Einhorn 1984, Delloye et al. 1985, Munting et al. 1988) or particles (Iwata et al. 1981, Bolander and Baslian 1986, Gepstein et al. 1987, Köhler and Kreicbergs 1987) might improve the healing of diaphyseal segmental defects. However, unless a standard bone graft is compared with identically processed, but also demineralized bone, conclusions are unreliable. Gendler (1986) introduced another variable by micro-perforating solid demineralized cortical bone.

Authors disagree on whether or not ethylene oxide sterilization impairs osteoinduction (Munting et al. 1988, Aspenberg et al. 1990, Urist 1968, Köhler and Kreicbergs 1987, Zislis et al. 1989, Moore et al. 1990).

Our study, using a rabbit diaphyseal bone defect model, was done to compare the healing of freeze-dried, ethylene oxide sterilized, segmental, allogenic bone grafts with the five variables perforation/nonperforation, demineralization/nondemineralization, and silicone rubber (Silastic) sheet exclusion from soft tissue.

## Materials and methods

### *Preparation of bone grafts*

18 adult, female, New Zealand white rabbits were used. 3 animals were used as donors. Lower extremity bones were harvested bilaterally and cut into 20-mm diaphyseal segments. Soft tissues were removed by sharp dissection and gross marrow elements by water jet. 15 bone segments were obtained, and 9 were multiply microperforated using 350- $\mu$  drill bits at slow speed (Gendler 1986). All the segments were washed in deionized water and defatted with ethyl alcohol and diethyl ether. 6 perforated and three nonperforated segments were demineralized with 3 decantings of 0.5 N HCl during continuous magnetic stirring for 24 h at 4 °C.

All the bone segments were placed in small combination plastic and polytetrafluorethylene (Ty-Vek) bags and gas sterilized with 12 percent ethylene oxide at room temperature (24 °C) for 14 h. Multiple cycles of flooding and purging with filtered room air, modifying the manufacturer's specification (VerTis Console #24, Gardiner, NY), was followed by freeze-drying for 5-8 days. This resulted in a final gravimetric water content of 1-2 percent, an average parts per million of 5.1 for ethylene oxide, 14.8 for ethylene 2-chlorhydrin, and 1,139 for ethylene glycol.

### Implantation, harvest, and evaluation

15 quadriceps injections of 50 mg/kg ketamine HCl (100 mg/mL) and 10 mg/kg Zylazine (20 mg/mL) were given. The right forearm was shaved, prepared with Betadine, and draped with sterile towels. A lateral incision was made and dissected to the ulna, disregarding the periosteum. With a power saw ("Minisaw" Synthes Inc, Paoli, Penn., U.S.A.), 18-mm midulnar segments were excised under cool-water irrigation. The saw blade was 1 mm thick, so the 20-mm bone grafts fitted snugly in the diaphyseal defects. Bone contact without angulation or gapping was thus achieved.

According to the preparation of the bone grafts, five groups of 3 animals were studied:

Group 1: Undemineralized—unperforated grafts

Group 2: Undemineralized—perforated grafts

Group 3: Demineralized—unperforated grafts

Group 4: Demineralized—perforated grafts

Group 5: Demineralized—perforated grafts, then enclosed by silicone rubber sheets (Silastic®, Dow-Corning, Midland, Michigan).

Immediately postoperatively, biplanar, high-resolution radiographs ("Faxitron" Hewlett-Packard) were taken to check implant position; in addition, these were repeated at harvest to evaluate radiographic healing (Figure 1).

The mean weights of the animals were 4.05 kg initially and 3.95 kg at death. There were no fractures or infections, and all the animals completed the study. Weight bearing generally began on the third day, and no medication for pain was given. At 18 days, the rabbits were weighed, killed, and their forearms were disarticulated at the elbow. The soft tissues were excised by sharp dissection, and the ulnas were dissected from the radii. It was noted whether or not the ulnas demonstrated resistance to torque upon simple manual twisting, after which they were placed in 10 percent neutral formalin for 1 week. The ulnas were then repeatedly washed with tap and deionized water. All the specimens were then demineralized in 20 percent formic acid for 8 days and again multiply rinsed. After dehydration in three decantings of ethyl alcohol, they were embedded in nitrocellulose for serial longitudinal sectioning and staining with hematoxylin and eosin.

Bone and cartilage formation was assessed by subjective evaluation by 2 observers (EG and GJM), whose conclusions were in agreement.

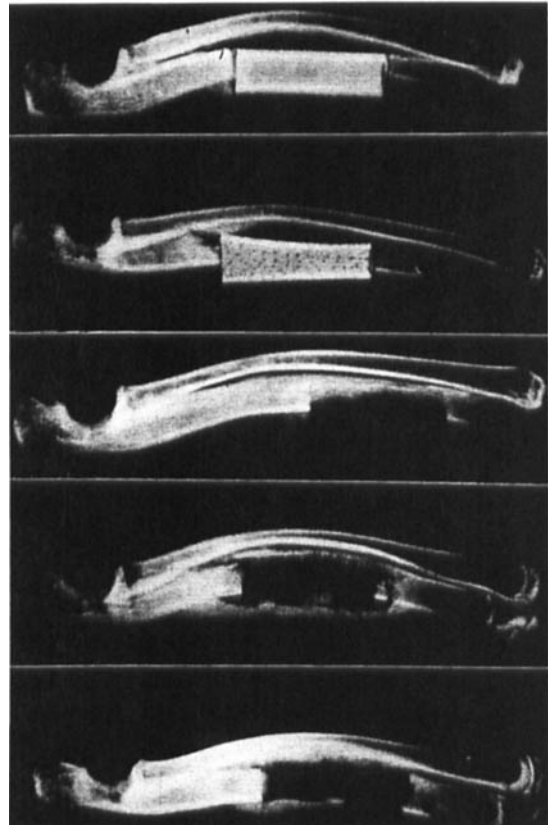


Figure 1. Lateral radiographs of the five groups of implants at 18 days, in descending numerical order with Group 1 at the top. Note failure to completely bridge in Groups 1 and 2, and partial mineralization of the initially radiolucent Groups 3 and 4 grafts. Silastic-excluded implants, Group 5, did not mineralize, but bone formed outside the Silastic sheets.

### Results

The gross findings in the three identical implants in each group were remarkably uniform. Resistance to simple manual torque was encountered only in the unexcluded demineralized Groups 3 and 4.

### Radiographic evaluation

At 18 days, the grafts of Groups 1 and 2 had no changes in bone density or bridging callus (Figure 1). Grafts of Group 3 showed bridging callus and slightly increased density in the segments. The grafts of Group 4 had the best bridging callus and more advanced mineralization of the segment than in Group 3. Endosteal callus formation was best seen in these perforated demineralized grafts. Finally, there was no mineralization or bridging callus in the grafts of Group 5.

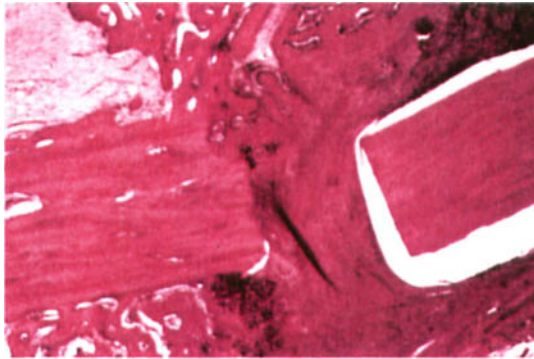


Figure 2. Junction between host bone on the left and undemineralized unperforated bone graft, Group 1, on the right. The graft is surrounded by an inflammatory capsule composed of collagen and leucocytes. Periosteal and endosteal callus has formed from the host bone but does not contact the implant. There is only fibrous tissue between the bone ends. HE x250.

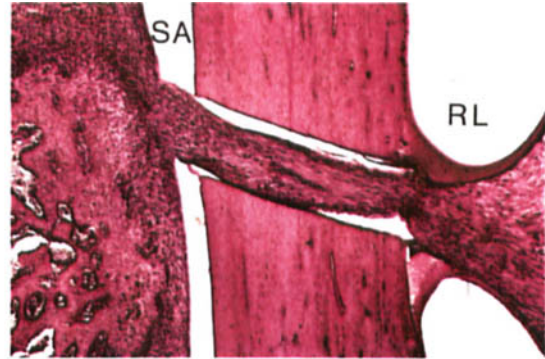


Figure 3. Mid-portion of a Group 2 undemineralized bone graft showing a longitudinal cut through a perforation. A strand of young collagen has invaded the hole, connecting the inflammatory capsule around the implant, but no cartilage or bone is formed. The graft lies in a false space due to a shrinkage artifact (SA) on the left, but on the right, fluid filled cavities with menisci of cellular debris are seen, which we have called "reactive lacunae" (RL). HE x250.

### Histologic evaluation

The groups of bone grafts were examined in terms of their response at the junctions, in their substance, and in the surrounding soft tissue.

*Group 1.* At the junctions between the unperforated undemineralized grafts and the host bone ends, there was mostly unoriented fibrous tissue (Figure 2). Abortive callus formed in a partial cuff of periosteally derived bone in some specimens, arising from the host bone ends, but not attached to the segments. These appeared to be standard centripetally oriented periosteal calluses, but they were separated from the graft by a reactive capsule of richly vascularized tissue. A less well-developed endosteally derived callus extended into the medullary canal of some of these implants; but, like the periosteal callus, was prevented from contacting the graft because of a reactive inflammatory membrane. The haversian canals were devoid of living cells, and the osteocytes were well preserved. The soft-tissue response to Group 1 was identical to that in Group 2, and is described below.

*Group 2.* At the junctions of the perforated undemineralized bone grafts with the host bone, the gaps were filled with fibrous tissue. No trabecular bone united the bone ends. Abortive periosteal and endosteal calluses were apparently prevented from contacting the implant by the same type of reactive capsule as in Group 1. The graft response was also the same as described for Group 1. Osteocytes were apparent, but microperforations and haversian canals were empty. The most obvious finding regarding the soft-tissue response was sequestration of portions of the bone-graft segments into "reactive lacunae" (Figure 3). These and the implants that lay within them

were surrounded by an inflammatory capsule of mononuclear leukocytes, which were most numerous at the bone surface. These "lacunae" often showed a meniscus of blood cells, proving they contained fluid, but there was no staining of proteinaceous content to suggest an exudate. On other surfaces of the implants, there were definite shrinkage artifacts. In almost all the microperforations, strands of fibrous tissue traversed the holes, but no cartilage or bone was formed.

*Group 3.* Most of the bone junctions between the unperforated demineralized grafts and the host were circumferentially bridged by new bone. Endosteal callus extended further into the medullary canal than in previous groups. The grafts were partially covered externally and internally by a layer of new bone and occasional areas of fibrocartilage (Figure 4). At the implant surfaces, there was patchy invasion of superficial haversian canals by cells that resembled viable chondroblasts. However, deeper in the matrix the haversian canals remained acellular. At the soft-tissue interface, the trabecular bone was so closely apposed to the implant surface that the bone graft changed gradually from bone into soft tissue without demarcation. In contrast to undemineralized bone, no surrounding inflammatory reaction was observed.

*Group 4.* The junctions of the perforated demineralized grafts with the host bone were essentially as described for Group 3. Trabecular bone bridging was observed in all the specimens, combining periosteal and endosteal callus formation (Figure 5). The implants were as described for the grafts in Group 3 regarding surface formation of new bone. However, there were two further striking findings. Almost all the perforations were filled with either chondrocytes

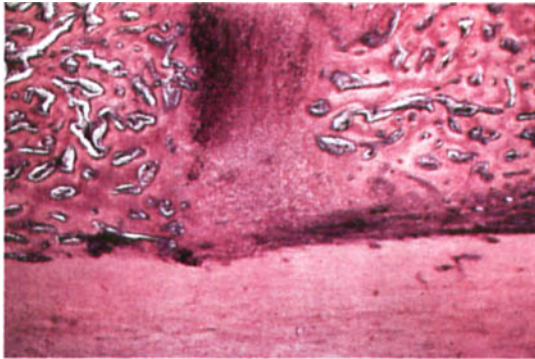


Figure 4. Bone formed on the surface of the mid-portion of an unperforated demineralized Group 3 graft. The upper two thirds is exuberant new bone closely apposed to the graft in the lower portion.

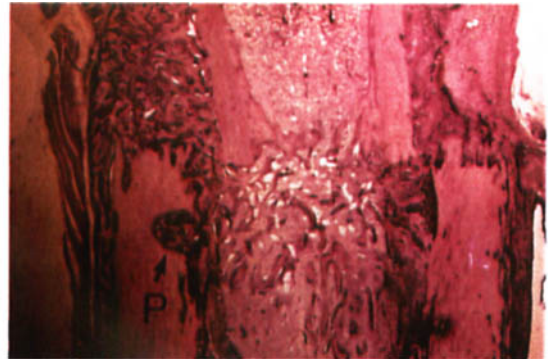


Figure 5. Junction between host bone above and demineralized perforated Group 4 bone graft below. Periosteal and endosteal callus are well developed and bridge from host to graft, blending with new bone formed by the graft. A perforation (P) filled with cells is visible in the implant. HE  $\times 50$ .



Figure 6. In this demineralized Group 4 graft, a transversely cut perforation contains bone trabeculae (small arrow); an obliquely cut perforation (large arrow) contains chondrocytes, so at 18 days they have become centers of osteoinduction. HE  $\times 500$ .

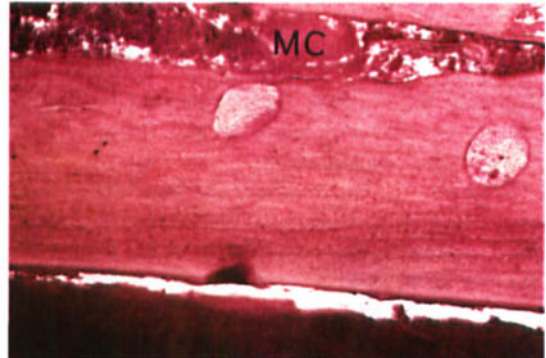


Figure 7. Mid-portion of a perforated demineralized graft excluded from soft tissue by a silastic sheet, Group 5. The medullary canal (MC), perforations, and space between the sheet and the graft (lower edge), are filled with necrotic cells. Silastic prevented periosteal bridging. HE  $\times 100$ .

and enchondral bone or with trabecular bone (Figure 6). A few perforations were seen to be enlarged, indicating that bone resorption was beginning at 18 days. Throughout most of the implanted segments, haversian canals were massively populated with cells resembling chondroblasts. The soft tissues were closely apposed to the surfaces of the grafts so as to almost completely incorporate them.

*Group 5.* These were perforated demineralized segments that were separated from the surrounding soft tissues by silicone rubber (Silastic) sheets. At the bone junctions the gaps were filled with primitive connective tissue, which invaded the medullary canals for several hundred microns and then became necrotic. The graft was necrotic except for a small part immediately adjacent to the host bone. A few haversian canals and perforations were invaded by chondroblasts in

these regions, but no bone was formed. The medullary canals and the perforations contained cell detritus (Figure 7). Between the Silastic sheets and the grafts, there was a mass of homogeneous eosinophilic material containing occasional pycnotic nuclei suggesting a necrotic inflammatory infiltrate.

## Discussion

The ability of demineralized bone to induce new bone has been studied for over 25 years (Urist 1965). Several investigators have confirmed the concept of bone induction due to a protein with morphogenic ability—called bone morphogenetic protein, or BMP (Urist et al. 1987)—and its origin from properly demineralized

bone (Nade and Burwell 1977, Tuli and Singh 1978, Oikarinen and Kahonen 1979, Iwata et al. 1981, Delloye et al. 1985, Saveliev and Sivkov 1986, Köhler and Kreicbergs 1987, Gepstein et al. 1987). There are three basic biochemical techniques that are used in the processing of demineralized bone for osteoinductive purposes. They are the methods of Urist (1987), Reddi and Huggins (1985), and Gendler (1986). Because different techniques and agents are used, it is reasonable that each matrix differs from the others, and thus exhibits different characteristics, including osteoinductivity (Delloye et al. 1985, Hollinger et al. 1991).

Undemineralized grafts, Groups 1 and 2, showed shrinkage artifacts, suggesting that the soft tissues were only loosely adherent to the implants. The "reactive lacunae" spaces often contained a cellular meniscus, proving they were fluid-filled. Both changes reflect an inflammatory response. Urist (1975) reported this phenomenon with undemineralized particles of bone 4 weeks after implantation. He called the cavities "sterile abscesses," but did not mention them in connection with solid undemineralized implants. These shrinkage artifacts and reactive lacunae sharply contrasted with the intimate incorporation of demineralized Group 3 and 4 grafts into their soft-tissue envelopes. Antigenic reaction to the undemineralized bone is a possible reason for the different modes of graft incorporation.

Although microperforations in undemineralized bone were penetrated by fibrous tissue, neither cartilage nor bone was formed. In contrast, microperforations in the demineralized bone became centers of osteoinductivity (Gendler 1986). This supports the concept of a cascade of osteoinduction, first described by Urist (1968) and later simplified by Reddi (1985). Once demineralized bone is populated by osteoprogenitor cells, matrix replacement proceeds rapidly (Gendler 1986, Bernick 1989), and centrifugally, in contrast to osteoconductive replacement.

Microperforations became centers of osteoinduction, and they provided access for permeation by haversian canals, setting the stage for more rapid replacement of the graft. Necrosis of the excluded segments was due to denial of access of blood vessels to the bone graft.

The silicone rubber (Silastic) sheet limited the active chemotaxis of mesenchymal cells, which was observed when soft tissues were allowed to contact the demineralized bone matrix. Ethylene oxide gas sterilization did not impair osteoinduction, confirming our previous experience (Moore et al. 1990). This statement is supported by two observations. First, comparison of the rate of bone formation with that of others who did not use ethylene oxide sterilization in their solid (Tuli and Singh 1978, Vandersteenhoven and

Spector 1983, Bernick et al. 1989) or powdered (Reddi 1985) implants, shows ethylene oxide sterilized Gendler matrix to form new bone at least as rapidly, if not more rapidly, than the others. Secondly, a major factor in reported suppression of osteoinduction is the presence of highly inflammatory ethylene oxide residues in the matrix. Zislis (1989) found that BMP activity is not impaired if the matrix is adequately aerated. Until the degree of residue removal and other relevant variables are studied and quantitated, unsupported general statements (Munting et al. 1988, Aspenberg et al. 1990) about the effect of ethylene oxide on osteoinduction cannot be considered reliable.

Most histologic studies have documented diaphyseal healing with demineralized bone matrix at 6 to 16 weeks (Tuli and Singh 1978, Köhler and Kreicbergs 1987). One of the most important factors in healing of long-bone defects is whether or not mechanically stabilizing callus unites the bone ends in the early stages of healing. Our observations lead us to conclude that there are differences in the quality of early bridging using different graft preparations; and demineralized bone grafts, particularly when microperforated, are superior to undemineralized grafts for this purpose.

## Acknowledgements

Dr. Sigholm was an Elinor Barry Research Fellow at Orthopedic Hospital for the academic year of 1987-1988 with additional support and funding from the Department of Orthopedics of the University of Southern California, Pacific Coast Tissue Bank, and Pacific Coast Transplant Laboratory—all in Los Angeles, CA, U.S.A.

## References

- Aspenberg P, Johnsson E, Thorngren K G. Dose-dependent reduction of bone inductive properties by ethylene oxide. *J Bone Joint Surg (Br)* 1990; 72 (6): 1036-7.
- Bernick S, Paule W, Ertl D, Nishimoto S K, Nimni M E. Cellular events associated with the induction of bone by demineralized bone. *J Orthop Res* 1989; 7 (1): 1-11.
- Bolander M E, Baslian G. The use of demineralized bone matrix in the repair of segmental defects. Augmentation with extracted matrix proteins and a comparison with autologous grafts. *J Bone Joint Surg (Am)* 1986; 68 (8): 1264-74.
- Delloye C, Hebrant A, Munting E, Piret L, Coutelier L. The osteoinductive capacity of differently HCl decalcified bone alloimplants. *Acta Orthop Scand* 1985; 56 (4): 318-22.
- Einhorn T A, Lane J M, Burstein A H, Kopman C R, Vigorita V J. The healing of segmental bone defects induced by demineralized bone matrix. A radiographic and biomechanical study. *J Bone Joint Surg (Am)* 1984; 66 (2): 274-9.

- Gendler E. Perforated demineralized bone matrix: A new form of osteoinductive biomaterial. *J Biomed Mater Res* 1986; 20, (6): 687-697.
- Gepstein R, Weiss R E, Hallel T. Bridging large defects in bone by demineralized bone matrix in the form of a powder. A radiographic, histological, and radioisotope uptake study in rats. *J Bone Joint Surg (Am)* 1987; 69 (7): 984-92.
- Hollinger J O, Mark D E, Goco P, Quigley N, Desverreaux R W, Bach D E. A comparison of four particulate bone derivatives. *Clin Orthop* 1991; 267: 255-63.
- Iwata H, Hanamura H, Kaneko M, Yasuhara N, Terashima Y, Kajino G, Ida K, Mabuchi Y, Nakagawa M. Chemosterilized autolysed antigen-extracted allogeneic (AAA) bone matrix gelatin for repair of defects from excision of benign bone tumors. *Clin Orthop* 1981; 154: 150-5.
- Köhler P, Kreicbergs A. Incorporation of autoclaved autogeneic bone supplemented with allogeneic demineralized bone matrix. An experimental study in the rabbit. *Clin Orthop* 1987; 218: 247-58.
- Moore T M, Artal R, Arenas M, Gendler E. Influence of post-mortem time and temperature on osteoinductive activity of demineralized microperforated ethylene oxide sterilized syngeneic bone implant in the rat. *Clin Orthop* 1990; 259: 239-44.
- Munting E, Wilmart J F, Wijne A, Hennebert P, Delloye C. Effect of sterilization on osteoinduction. Comparison of five methods in demineralized rat bone. *Acta Orthop Scand* 1988; 59 (1): 34-8.
- Nade S, Burwell R G. Decalcified bone as a substrate for osteogenesis. An appraisal of the interrelation of bone and marrow in combined grafts. *J Bone Joint Surg (Br)* 1977; 59 (2): 189-96.
- Oikarinen J, Korhonen L K. Repair of bone defects by bone inductive material. *Acta Orthop Scand* 1979; 50 (1): 21-6.
- Reddi A H. Implant-stimulated interface reactions during collagenous bone matrix-induced bone formation. *J Biomed Mater Res* 1985; 19 (3): 233-9.
- Saveliev V I, Sivkov S. Our experience with supply of demineralized bone grafts. *Ortoped Travmat Protezirovaniye (USSR)* 1986; 8: 22-5.
- Tuli S M, Singh A D. The osteoinductive property of decalcified bone matrix. An experimental study. *J Bone Joint Surg (Br)* 1978; 60 (1): 116-23.
- Urist M R. Bone formation by autoinduction. *Science* 1965; 150 (698): 893-9.
- Urist M R. Surface decalcified allogenic bone (SDAB) implants: A preliminary report of 10 cases and 25 comparable operations with undecalcified lyophilized bone implants. *Clin Orthop* 1968; 56: 37-50.
- Urist M R. A chemosterilized antigen-extracted autodigested alloimplant for bone banks. *Arch Surg* 1975; 110: 416-28.
- Urist M R, Chung J J, Lietze A, Huo Y K, Brownell A G, DeLange R J. Methods of preparation and bioassay of bone morphogenetic protein and polypeptide fragments. *Meth Enzymol* 1987; 146: 296-312.
- Vandersteenhoven J, Spector M. Histological investigation of bone induction by demineralized allogenic bone matrix: a natural biomaterial for osseous reconstruction. *J Biomed Mater Res* 1983; 17: 1003-14.
- Zislis T, Martin S A, Cerbas E, Heath J R, Mansfield J L, Hollinger J O. A scanning electron microscopic study of in vitro toxicity of ethylene oxide-sterilized bone repair materials. *J Oral Implant* 1989; 15: 41-6.