

# The osteoinductive capacity of differently HCl-decalcified bone alloimplants

Three procedures to obtain bone inductive implants were tested heterotopically in 3-month-old allogeneic rats: 1) antigen-extracted HCl-decalcified at 4°C, autolysed implant (AAA bone); 2) HCl-decalcified implant at 4°C; 3) HCl-decalcified implant at room temperature. Each type of implant was either deep-frozen at -35°C for at least 2 months or immediately freeze-dried. The bone inductive capacity of the differently HCl-decalcified cortical bone implant was evaluated at 2 months by isotopic strontium incorporation and by ash-weight measurements. Bone HCl-decalcification alone, either at 4°C or at room temperature, gave a higher new bone yield than the freeze-dried AAA bone. The type or short-term preservation technique had no effect on the osteoinductive capacity of either of the differently treated implants, AAA bone expected.

Correspondence: Orthopaedic Research Laboratory, Faculty of Medicine, University of Louvain, Tour Pasteur 5388, 53 av. Mounier – B. 1200, Brussels, Belgium

**Christian Delloye**  
**Alain Hebrant**  
**Everard Munting**  
**Louis Piret<sup>1</sup>**  
**Leopold Coutelier**

Orthopaedic Research Laboratory, Department of Orthopaedic Surgery and <sup>1</sup>Nuclear Medicine Department, University of Louvain, Brussels, Belgium

Since the first reports of osteogenesis in heterotopic HCl-decalcified matrix (Urist 1965, Van de Putte & Urist 1965), the validity of this bone-inducing system has been further confirmed. Several studies have shown that an excellent osteoinductive response was obtained with HCl-decalcified cortical bone, either at 4°C (Urist 1965, Van de Putte & Urist 1965, Koskinen et al. 1972, Oikarinen & Korhonen 1979, Glowacki et al. 1981, Van der Steenhoven & Spector 1983) or at ambient temperature (Narang et al. 1971, Chalmers et al. 1975, Tuli & Singh 1978, Gupta & Tuli 1982, Einhorn et al. 1984).

In 1975, Urist proposed combined chemosterilization, antigen-extraction and autodigestion to obtain bone alloimplant (AAA bone). This procedure theoretically offered the possibility of obtaining a sterile, low antigenic bone matrix containing a high activity level of the bone morphogenetic protein (BMP). However, the 5 days required to process the samples render its use difficult in routine bone-banking procedures.

These differently HCl-decalcified implants have been proposed as possible substitutes to

autografts for treatment of bone defects (Urist 1968, 1983, Tuli & Singh 1978, Glowacki et al. 1981, Urist & Dawson 1981, Oikarinen 1982, Volpon et al. 1982, Einhorn et al. 1984). BMP transferred by such implants would be the principal induction effector (Urist 1983).

Freeze-drying and deep-freezing are the most widely accepted techniques for bone preservation. None of the above-mentioned studies compared the different procedures of bone HCl-decalcified and the mode of preservation of such implants. Our aim was to compare quantitatively the osteoinductive potential of AAA bone to cortical bone decalcified with HCl either at 4°C or at ambient temperature. The effects of short-term deep-freezing drying on implant osteoinduction were also evaluated.

## Material and methods

### Implant preparation

Left and right femoral shafts freed from soft tissues, periosteum and bone marrow were obtained from 45 inbred 3-month-old female Lou/Dec Wistar rats weighing 180–200 g. The time elapsing between the harvest of the shafts and the beginning of the extrac-

tion procedures never exceeded 3 h. Three procedures of implant preparation were tested. The first, (P<sub>1</sub>) was according to the method of Urist (1980). In brief, shafts were defatted in a chloroform-methanol (1/1) solution for 4 h, and subsequently demineralized in 0.6N HCl at 4°C for 12 h. After trimming to 6-mm-long segments, the diaphyses were rinsed in distilled H<sub>2</sub>O and then immersed for 3 days at 37°C, in 0.1M NaHPO<sub>4</sub> solution buffered at pH 7.4 with 0.1N NaOH and containing 10 mM iodoacetic acid and 10 mM sodium azide. The antigen-extracted autolysed allogeneic implants (AAA bone) thus obtained were either freeze-dried for 2 days and kept in a vacuum at room temperature or deep-frozen at -35°C for at least 2 months.

The second procedure (P<sub>2</sub>) was as follows. The cleaned cortical femoral shafts were immediately demineralized in 0.6N HCl at 4°C for 12 h, thoroughly rinsed in distilled H<sub>2</sub>O and trimmed to 6-mm-long segments. These implants were also freeze-dried or deep-frozen as described for the first group.

For the third procedure (P<sub>3</sub>), the shafts were demineralized in 0.6N HCl at room temperature (20–25°C) for 4 h, and carefully rinsed with distilled H<sub>2</sub>O. After trimming to 6-mm-long diaphyseal segments, the implants were freeze-dried or deep-frozen as above.

The first procedure (P<sub>1</sub>) will be referred to below as the multi-step procedure, whereas the second (P<sub>2</sub>) and the third (P<sub>3</sub>) will be referred to as the one-step procedures.

### Implantation procedure

The freeze-dried implants were reconstituted by immersion of the samples in distilled H<sub>2</sub>O for 1 h at room temperature. The deep-frozen implants were allowed to thaw for a period of 1 h at room temperature. Before implantation, each specimen was sterilized by immersion for 5 h in 0.1% Thimerosal in isotonic saline (Federa Laboratories, Brussels, Belgium). The sterilant was washed away immediately before the surgical procedure by rinsing the implants several times in sterile saline.

For the present study, 45 inbred 3-month-old female Wistar rats were used as allogeneic recipients. Fifteen animals per set of experiments, repeated 3 times, were distributed according to a balanced incomplete block design (Cox & Cochran 1957) in which each rat, representing one block, received two differently treated implants: one placed in the right paravertebral muscle, and the other in the left. The animals were fed, *ad libitum*, on regular rat chow. They were sacrificed 2 months after the implanta-

tion. Implants were dissected from the surrounding muscle under a magnifying lens, carefully blotted, dried for 1 h at ambient temperature, and weighed on an analytical balance by the same observer.

### Analytical procedures

Implant new bone formation at the eighth week was assessed by intraperitoneal injection of 10 µCi of Sr<sup>85</sup>Cl<sub>2</sub> per 100 g of body weight, 3½ days before sacrifice. One ilium per animal, carefully dissected free from adherent connective tissue and weighed, served as control of the host skeleton activity. Radioactivity of each recovered sample was counted in a Packard model 3385 gamma counter (Packard Instruments, Downers Grove, Ill., USA). Background readings were subtracted and data were adjusted to give counts per min per mg of sample. By relating the implant activity to the ilium activity, an osteogenic index (I) was calculated according to Elves (1974). Neperian logarithmic transformation was used to account for data means and variance proportionality:

$$\ln I = \ln \left[ \frac{\text{Counts/min/mg implant}}{\text{Counts/min/mg ilium}} \times 100 \right]$$

The mineral content of the recovered implants was assessed by ashing at 700°C for 8 h and weighing the inorganic residue on an analytical balance. Ash weights were expressed as a percentage of the recovered blotted implant weight (mineral fraction) and in absolute value (mg). In a set of preliminary experiments, 20 femoral diaphyses (6-mm-long) from rats of the same origin were decalcified either at 20°C for 4 h for 12 h. The mean dry weight after demineralization as obtained by drying at 60°C for 2 h was, respectively, 10.69 ± 1.35 mg and 10.72 ± 2.24 mg. The mean ash residue was less than 1 percent of the demineralized segment dry weight in both procedures.

The presence of new bone in the different organic matrices 2 months after implantation, was also assessed by a histological method. For this purpose, 12 females, of the same age and strain as those used in the aforementioned studies, were randomly divided into six equal groups. Each group received an implant from either one of the described procedures in one paravertebral muscle. The opposite muscle, serving as control, was sham-operated or received a small polyethylene tube. After sacrifice, the implants were demineralized, embedded in paraffin and stained with haematoxylin-eosin. Sections were examined for the presence of new bone, cartilage, old matrix, bone marrow and inflammatory cells.

## Statistics

Statistical analysis, adapted to the balanced incomplete block design, was based on the adjusted treatment means to allow for the fact that not every treatment occurred in every block (Cox & Cochran 1957). The difference among treatments was tested by a two-way variance analysis (F-test), after having verified that variance within each treatment was similar. Multiple comparisons between adjusted treatment means were performed by *t*-test, using the residual variance and by contrast analysis.

## Results

Table 1 lists for each quantitative evaluation, the mean value and its one standard deviation (first row) and their adjusted treatment mean (second row).

There was a marked difference in the Sr85 osteogenic index among the six groups ( $P < 0.001$ ). Furthermore, multiple comparison of treatments showed that in the one-step procedures, whether at 4° or 20°C, isotope uptake was similar at 2 months and was significantly higher than in the multi-step procedure ( $P < 0.01$ ). In deep-frozen, preserved AAA implants, strontium incorporation was superior to the freeze-dried implants ( $P < 0.05$ ). No difference was found between the two modes of preservation in the one-step procedures ( $P > 0.05$ ). After 2 months of implantation, the percentage of the mineral fraction in the various groups was different ( $P < 0.01$ ). Here also, adjusted means of implants that were only decalcified, whether at 4°C or at room temper-

ature, were similar and higher than in the AAA group ( $P < 0.01$ ). No difference was observed between the mineral fractions of deep-frozen and freeze-dried implants in each procedure. The difference in the absolute ash weight among the six groups was also highly significant ( $P < 0.005$ ). Multiple comparisons showed that implants, HCl-decalcified at 20°C gave higher results than those decalcified at 4°C and the deep-frozen AAA bone ( $P < 0.01$ ). Freeze-dried AAA bone implants had significantly the lowest mineral content when compared to the others ( $P < 0.05$  and 0.01).

Histologically, living bone lines by active osteoblasts were constantly observed inside the various old bone matrices and in their original diaphyseal medullary cavity. The shape of the new bone which contained bone marrow tended to be ossicular. In all cases, a few cartilaginous cells were found, disseminated through the old matrix. A thin fibrous tissue envelope surrounded the implants, and inflammatory cell infiltrate was very slight or even absent. No histological difference in the amount of induced new bone could be found among the differently processed implants, except for a slight decrease in the AAA group. New bone was not observed around the plastic tubes or in the sham-operated muscles.

## Discussion

Decalcification at room temperature is at least as effective for inducing new bone as decalcification at 4°C. Urist could not find a difference

Table 1. Quantitative evaluation and multiple comparisons after 2 month implantation.

		AAA bone (P1)		HCl-decalcified 4°C (P2)		HCl-decalcified 20°C (P3)	
		FD	DF	FD	DF	FD	DF
Osteogenic index (1)	A	4.57±0.55	4.99±0.44	5.32±0.64	5.20±0.52	5.13±0.35	5.26±0.47
	B	4.62	4.92	5.22	5.22	5.23	5.24
Mineral fraction (%) (2)	A	16.4±5.1	16.3±7.1	19.1±4.9	21.1±3.9	19.7±3.8	21±4.8
	B	16.2	16.6	19.1	20.8	20.0	20.9
Absolute ash weight (mg) (3)	A	4.42±1.81	5.36±1.89	5.21±1.76	5.58±1.54	6.62±1.76	6.81±1.28
	B	4.36	5.41	5.22	5.55	6.68	6.76

A) Values are treatment means ± 1 standard deviations. B) Adjusted treatment means. The residual standard deviations, free from animal and treatment effects and generated from the two-way variance analysis were respectively ± 0.33 (1) ± 4.01 (2), ± 1.71 (3). For the multiple comparisons, the significance of the difference between adjusted means was assessed by *t*-test and contrast analysis. \* $p < 0.05$ ; \*\* $p < 0.01$ . P1 = Multi-step procedure; P2–P3 = One step procedures; FD = Freeze-dried; DF = Deep-frozen.

between the two temperatures provided that demineralization at 20°C lasted no longer than 3 days (Urist et al. 1968). After such a period, bone induction decreased rapidly. As for strontium incorporation, ash residues of AAA bone compared unfavourably with the one-step processed specimens, except for the absolute ash weight in the deep-frozen specimens.

The amount of induced new bone is dependent on the age of the recipient (Irving et al. 1981) and the source of bone implants (Urist et al. 1970, Lindholm & Urist 1980). Strontium uptake into the iliac bone has also been demonstrated to be age-dependent (Elves 1974). Our results were free of such variation because those conditions were taken into consideration.

Freeze-drying and deep-freezing are the two standard preservation techniques in bone-banking procedures. In the AAA group, deep-freezing preservation elicited significantly better results than freeze-drying. However, no difference could be found between deep-frozen and freeze-dried implants which have been decalcified either at 4°C or at room temperature. The two techniques appear to be equally effective in the short term at preserving the BMP retained in these one-step-processed bone cortical implants. A similar conclusion was previously drawn by Nade & Burwell (1977), although it concerned decalcified cancellous implants. For longer preservation, unpublished data indicate that freeze-dried implants which were HCl-decalcified at 4°C remained osteoinductive after more than 10 years.

Histology was performed in a separate set of animals to ascertain the presence of induced new bone in the various implants. Inside the different residual matrices, new trabecular bone, limited by a thin cortex and containing bone marrow, was present. This histologic pattern has also been previously reported (Urist 1965, van de Putte & Vrist 1965, Koskinen et al. 1972, Chalmers et al. 1975, van der Steenhoven & Spector 1983). Qualitative examination of the samples did not elicit any obvious difference in the amount of induced bone, except in the AAA group where it tended to be lower. However, the number of histological samples was too small to allow a statistical histomorphometrical assessment.

In an earlier report, Oikarinen & Korhonen

(1979) used semiquantitative histological evaluation to compare the healing of cold HCl-decalcified implants with AAA bone orthotopically implanted in rabbits. The bone-defect-repairing capacity of AAA implants was not found superior to implants that were only decalcified.

It should be pointed out that the induced bone yield is lower in higher vertebrates (Urist et al. 1975) and that the osteoinductive value we obtained in our study for the different procedures in this heterotopic model may be influenced by a different environment such as in orthotopic conditions. Moreover, in long-lived animals the immune response may be a more prevailing factor influencing new bone formation. In the rat, preserved allograft incorporation as assessed by histologic scores has been shown to be apparently unaffected by the presence of even a strong transplantation barrier (Dollinger et al. 1984). On the other hand, allografting a frozen implant in an unmatched recipient in the dog significantly impaired bone healing (Bos et al. 1983, Powell et al. 1983). Consequently, the value of AAA bone with its claim to low antigenicity when allotransplanted in long-lived animals needs further study and comparison with non-autodigested HCl-decalcified implants.

### Acknowledgements

We wish to express our gratitude to B. Govaerts and M. Arnould for the statistical analysis and to E. E. Delvin (Shriners Hospital, McGill University) for reviewing the manuscript.

This work was supported by grants from the Fonds National de la Recherche Scientifique and the Société Générale de Banque (Belgium).

### References

- Bos, G. D., Goldberg, V. M., Powell, A. E., Heiple, K. G. & Zika, J. M. (1983) The effect of histocompatibility matching of canine frozen bone allografts. *J. Bone Joint Surg.* **65-A**, 89–96.
- Chalmers, J., Gray, D. H. & Rush, J. (1975) Observations on the induction of bone in soft tissues. *J. Bone Joint Surg.* **57-B**, 36–45.

- Cox, G. M. & Cochran, W. G. (1957) *Experimental designs*, 2nd ed., pp. 439–482. John Wiley and Sons, New York, London.
- Dollinger, B., Bos, G. M., Goldberg, J. M., Zika, J. M., Heiple, K. & Powell, A. (1984) The ultimate fate of fresh and frozen orthotopic bone allografts in genetically defined rats. *Trans. Orthop. Res. Soc.* **9**, 339.
- Einhorn, T. A., Lane, J. M., Burstein, A. H., Kopman, C. R. & Vigorita, V. J. (1984) The healing of segmental bone defects induced by demineralized bone matrix. *J. Bone Joint Surg.* **66-A**, 274–279.
- Elves, M. W. (1974) An evaluation of the use of strontium 85 for the assessment of experimental bone grafts. *Acta Orthop. Scand.* **45**, 641–651.
- Glowacki, J., Kaban, K. B., Murray, J. E., Folkman, J. & Mulliken, J. P. (1981) Application of the biological principle of induced osteogenesis for craniofacial defects. *Lancet* **i**, 959–963.
- Gupta, D. & Tuli, S. M. (1982) Osteoinductivity of partially decalcified alloimplants in healing of large osteoperiosteal defects. *Acta Orthop. Scand.* **53**, 857–865.
- Irving, J. T., LeBolt, S. A. & Schneider, E. L. (1981) Ectopic bone formation and aging. *Clin. Orthop.* **154**, 249–253.
- Koskinen, E. V. S., Ryppy, S. A. & Lindholm, T. S. (1972) Osteoinduction and osteogenesis in implants of allogenic bone matrix. *Clin. Orthop.* **87**, 116–131.
- Lindholm, T. S. & Urist, M. R. (1980) A quantitative analysis of new bone formation by induction in composite grafts of bone marrow and bone matrix. *Clin. Orthop.* **150**, 288–300.
- Nade, S. & Burwell, G. R. (1977) Decalcified bone as a substitute for osteogenesis. An appraisal of the interrelation of bone and marrow in combined grafts. *J. Bone Joint Surg.* **59-B**, 189–199.
- Narang, R., Lloyd, W. & Wells, H. (1971) Grafts of decalcified allogeneic bone matrix promote the healing of fibular fracture gaps in rats. *Clin. Orthop.* **80**, 174–180.
- Oikarinen, J. & Korhonen, L. K. (1979) The bone inductive capacity of various bone transplanting materials used for treatment of experimental bone defects. *Clin. Orthop.* **140**, 208–215.
- Oikarinen, J. (1982) experimental spinal fusion with decalcified bone matrix and deep-frozen allogeneic bone in rabbits. *Clin. Orthop.* **162**, 200–218.
- Powell, A. E., Bos, G. D., Goldberg, V. M., Zika, J. M. & Heiple, K. G. (1983) Immune responses to bone allografts. In: *Osteochondral allografts. Biology, banking and clinical applications*, (Eds. Friedlander, G. E., Mankin, M. J. & Sell, K.), pp. 141–150. Little Brown, Boston, Toronto.
- Tuli, S. M. & Singh, A. D. (1978) The osteoinductive property of decalcified bone matrix. An experimental study. *J. Bone Joint Surg.* **60-B**, 116–123.
- Urist, M. R. (1965) Bone: formation by autoinduction. *Science* **150**, 893–899.
- Urist, M. R., Dowell, T. A., Hay, P. H. & Strates, B. S. (1968) Inductive substrates for bone formation. *Clin. Orthop.* **59**, 59–96.
- Urist, M. R. (1968) Surface-decalcified allogeneic bone (SDAB) implants: a preliminary report of 10 cases and 25 comparable operations with undecalcified lyophilized bone implants. *Clin. Orthop.* **56**, 37–50.
- Urist, M. R., Jurist, J. M., Dubuc, F. L. & Strates, B. S. (1970) Quantification of new formation in intramuscular implants of bone matrix in rabbits. *Clin. Orthop.* **68**, 279–293.
- Urist, M. R., Mikulski, A. & Boyd, S. D. (1975) A chemosterilized antigen-extracted autodigested alloimplant for bone banks. *Arch. Surg.* **110**, 416–428.
- Urist, M. R. (1980) Bone transplants and implants. In: *Fundamental and clinical bone physiology*, (Ed. Urist, M. R.), pp. 331–368. J. B. Lippincott, Philadelphia, Toronto.
- Urist, M. R. & Dawson, E. (1981) Intertransverse process fusion with the aid of chemosterilized autolyzed allogeneic (AAA) bone. *Clin. Orthop.* **154**, 97–113.
- Urist, M. R. (1983) Chemosterilized antigen-extracted surface-demineralized autolyzed allogeneic (AAA) bone for arthodesis. In: *Osteochondral allografts. Biology, banking and clinical applications*, (Eds. Friedlander, G. E., Mankin, M. J. & Sell, K.), pp. 183–201. Little Brown, Boston, Toronto.
- Van de Putte, K. A. & Urist, M. R. (1965) Osteogenesis in the interior or intramuscular implants of decalcified bone matrix. *Clin. Orthop.* **43**, 257–270.
- Van der Steenhoven, J. J. & Spector, M. (1983) Histological investigation of bone induction by demineralized allogeneic bone matrix: a natural biomaterial for osseous reconstruction. *J. Biomed. Mater. Res.* **17**, 1003–1014.
- Volpon, J. B., Camillo, A. M. & Concalves, P. (1982) The use of decalcified granulated homologous cortical bone matrix in the correction of diaphyseal bone defects. An experimental study in rabbits. *Arch. Orthop. Traumat. Surg.* **99**, 199–207.