Acta Orthop. Belg., 2009, **75**, 103-109

ORIGINAL STUDY

The effect of Bone Morphogenetic Protein-7 (OP-1) and demineralized bone matrix (DBM) in the rabbit tibial distraction model

Johan LAMMENS, Jos NIJS, Evert SCHEPERS, Nadine ECTORS, Daniel LISMONT, Bert VERDUYCKT

From the University Hospitals, KU Leuven, Belgium

OP-1 (800 µg) or DBM (1900 mg) were implanted in a rabbit tibial distraction model, and healing was compared to a non treated control group. The limbs were harvested after ten weeks and examined using radiography, computerized axial tomography and histological analysis. Neither of the treatments showed a changed healing pattern. Densities as measured by CT scan were not increased and the only significant finding was an increased area of bone formation in the DBM treated group (65% increase as compared to the OP-1 group). These experimental results do not show an effect of these substances in this model of bone lengthening. They indicate that further studies are warranted to understand the process of bone formation and the working mechanisms of substances that potentially trigger bone healing.

Keywords : Bone Morphogenetic Protein ; demineralized bone matrix ; limb lengthening ; quantitative computerized tomography ; bone formation.

INTRODUCTION

Bone distraction is an excellent example of *in vivo* tissue engineering because massive cylinders of newly formed bone can be created, allowing limb lengthening or reconstruction of large defects (*15*).

The slow progressive distraction after osteotomy results in a remarkable capacity of self repair. It creates a completely new bone segment with indistinguishable anatomical morphology and identical mechanical characteristics (*11,12*). Even under favorable conditions of stable fixation, the biological regeneration process is a complex time consuming phenomenon of interaction between cells and a variety of intra- and extracellular matrix components (*22*). The complete *in vivo* orchestration of this cascade is far from elucidated but progressive insights in the complex molecular interactions open new perspectives for the enhancement of bone repair (*3*). Because healing indices can be very long (1-2 months/cm) any therapeutic intervention

This research was supported by grant number G.0340.01 from "Fonds voor Wetenschappelijk Onderzoek" and approved by the Ethical Committee of the Universitaire Ziekenhuizen KULeuven under number 01027.

■ Johan Lammens, MD, PhD, Orthopaedic Surgeon.
■ Jos Nijs, Scientific Collaborator.
Department of Orthopedic Surgery & Prometheus, Division of Skeletal Tissue Engineering, KU Leuven.
■ Evert Schepers, MD, PhD, Professor in Prosthetic Dentistry.
Department of Prosthetic Dentistry & Prometheus, Division of Skeletal Tissue Engineering, KU Leuven.
■ N. Ectors, MD, PhD, Pathologist.
Department of Pathological Anatomy, KU Leuven.
■ D. Lismont, Coordinator of Bone bank.
■ B. Verduyckt, Coordinator of Bone bank.
Bone Bank, KU Leuven.
Universitaire Ziekenhuizen Leuven, Katholieke Universiteit Leuven, Belgium.
Correspondence : Johan Lammens, MD, PhD, Department of Orthopedic Surgery, Universitair Ziekenhuis Pellenberg, Weligerveld 1, 3212 Pellenberg, Belgium.
E-mail : johan.lammens@uz.kuleuven.ac.be

reducing them would be extremely welcome as it would decrease morbidity, functional limitations and working incapacity.

Interesting candidates for the stimulation of the bone healing process are purely inductive substances such as the bone morphogenetic proteins (BMP's) or the combined conductive and inductive matrix of demineralized bone (DBM) (*4,5,24,25*). By stimulating the differentiation of mesenchymal cells into the osteoblastic and chondroblastic lineage, they may contribute to intramembranous bone formation and endochondral ossification. Especially BMP-2 and BMP-7 are subject of intensive research. So far experimental and clinical investigations have focused on the healing of nonunions and segmental defects, whereas only few studies have dealt with healing of fresh fractures or distraction bone healing (*6,8,10*). The readily osteoconductive and highly osteoinductive capacity of the allogeneic tissue graft demineralized bone matrix (DBM), especially the one prepared from intramembranous bone, is widely used in maxillofacial procedures, and is also gaining popularity in orthopaedic surgery (*23,27*). At present few studies in the field of BMPs or DBM focus on acute bone healing or lengthening. In the present experiment a rabbit tibial distraction model was used to evaluate the potential beneficial effects of BMP-7 (OP-1) and DBM on the bone formation process during limb lengthening as a preliminary set-up towards possible clinical applications.

METHODS

Animals

Thirty-six mature female New Zealand white rabbits with a weight between 2.5 and 3 kilograms were used for this experiment. All operations were performed according to the guidelines of the Ethical Committee of our institution. Under general anaesthesia using ketamine (Ketalar®-Pfizer) and medetomidin hydrochloride (Domitor®-Pfizer), a circular fixator with three 1.5 mm Kirschner wires on both the proximal and distal ring, tensioned to 90 kg was applied on the right hind limb (fig 1). A transverse tibial osteotomy was performed subperiosteally with a chisel after drilling multiple 1.5 mm holes. The level of the osteotomy was chosen at the lower half of the diaphysis, below the junction with the fibula, avoiding the need for a fibulotomy. In the control animals (n = 11) the wounds were simply closed. In the OP-1 treated group (n = 14) 800 µg of the protein (Osigraft – Stryker Biotech USA) was reconstituted with 1.5 ml of a 0.9% saline solution, resulting in a wet sand like mass that was carefully placed between and around both tibial fragments, after which the skin was sutured. The same strategy was used for the DBM group (n = 11) where 1900 mg of powder was applied locally. The DBM was prepared from rabbit tibial bone shafts. The cortical bone was ground into particles between 200 and 500 µm diameter. Demineralization was performed using a two steps process with 0.5M hydrochloric acid during 2 hours at 4° Celsius, followed by repeated wash out with sterile distilled water and final soaking in absolute ethanol for one hour. The powder was finally lyophilized and sterilized with 15 kGy gamma irradiation.

After a period of 5 days the tibia was lengthened by 1 mm daily till a distraction of 15 mm was obtained. The fixator was left in place till 10 weeks postoperatively, at which moment the animals were sacrificed using an overdose of a mixture of 20% methoxy butyramide, 5% methylene ammonium iodide and 0.5% ethanol hydrochloride (T61® – Hoechst).

Radiology and bone density

Plain radiographs were taken in anteroposterior and lateral direction immediately after the surgical procedure and further at 2-week intervals. An evaluation was made for the length of the regenerate and the presence of callus, studying the amount, distribution, density and corticalization of the newly formed bone (fig 2). The right tibia was scanned preoperatively at the level of the future osteotomy and at sacrifice through the middle of the distracted area using a pQCT system Stratec 960A. (Norland Stratec, Birkenfeld, Germany). Out of these areas 1.2 mm thick slices, obtained with a voxel size of $0.148 \times 148 \times 1.25$ mm³, were calculated for volumetric total bone density and total area of new bone formation using the specific software (Version 5.2) (*14*) (fig 3).

Statistical Analysis

One sample t-test was used to evaluate the paired differences in pre- and postoperative results of all studied variables. The Wilcoxon unpaired t-test was used for comparing the three groups while the statistical significance level was set at 5%.

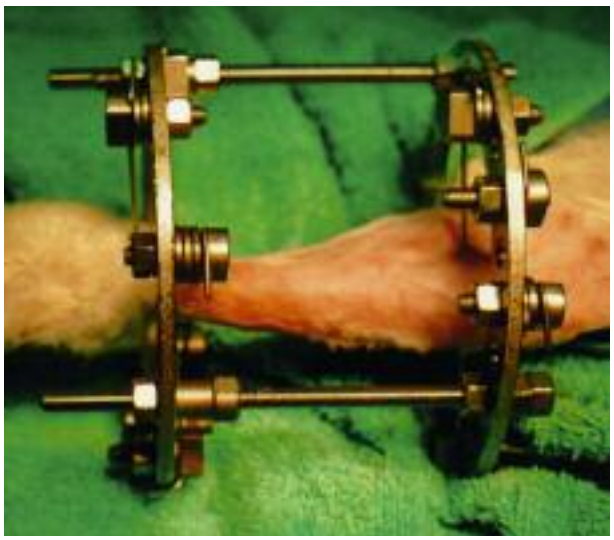

Fig. 1. — Ilizarov fixator with custom-made rings applied to the rabbit's tibia.

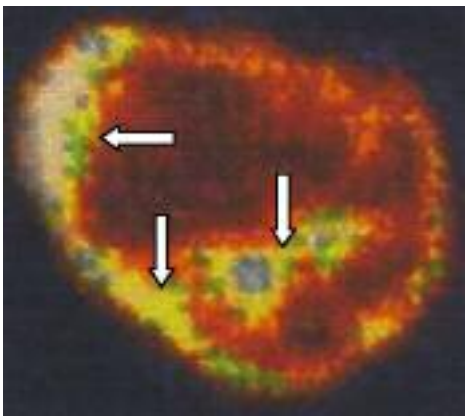

Fig. 3. — Quantitative CT scan with bright colored areas (yellow-white) indicating mature bone with density above 1000 mg/cm^3

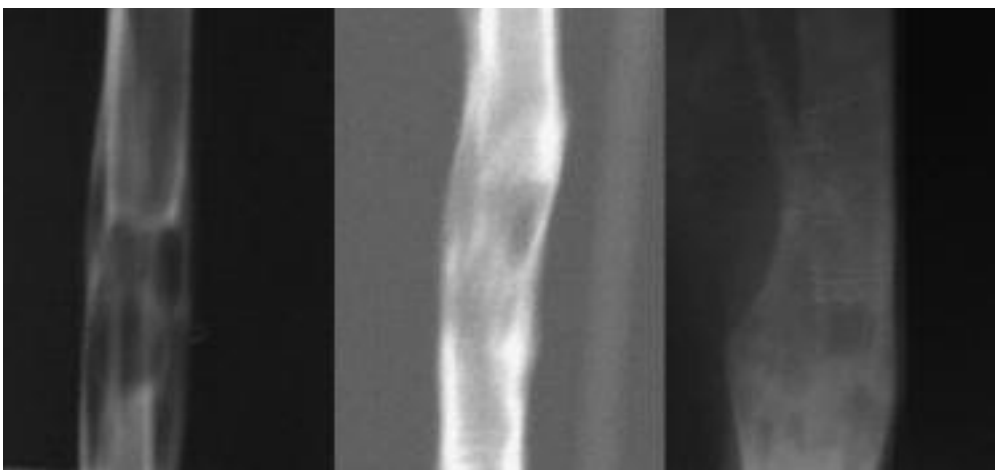

Fig. 2. — Standard radiographs of control (1), OP-1 (2) and DBM (3) treated animals at 10 weeks postoperatively. Comparable findings in the three groups, with slight fusiform increase in volume in the DBM group.

Histology

The lengthened specimens were processed for undecalcified sections. After fixation in a solution of formaldehyde neutralized with CaCO3 and ethanol, they were dehydrated in different concentrations of alcohol and finally embedded in methylmethacrylate. Transverse sections of 100 µm were cut with a microtome and polished to a thickness of 50-60 µm using a Minimet instrument and stained using a combination of Gieson picrofuchsine and Stevenel's Blue. The sections were evaluated for the quality of bony union and the cortical bridging.

Table I. — CT data for bone density.
Mean values and standard deviations for pre- and postoperative density and callus area, showing a significant decrease in density but increase in area for all groups (one sample t-test). The density loss between treated and control animals showed no significant difference, the area increase was only significant for the DBM treated group (Wilcoxon unpaired t-test).

	Total bone *Pre-operative*		Total bone *Post-operative*		% paired difference	
Group	mg/cm³	mm²	mg/cm³	mm²	density	Area
Control	760.4(153)	41.5(10)	379.9(134)	98.6(39)	52.5(24)*	242.7(95)*
OP-1	794.2(62)	34.7(2)	302.8(75)	74(16)	38.1(9)*	212.4(39)*
DBM	804.5(39)	37.4(4)	299.2(79)	103.3(25)	37.1(9)*	277.9(69)*°

Total bone (SD), * $p < 0.01$, ° $p < 0.05$.

RESULTS

Radiographic features and density measurements

On the radiographs a similar progression in callus formation was noted in all groups. As from the second week, signs of new bone formation were visible. Gap size was 15 mm at the end of the distraction in all animals. The distracted area progressively filled with new bone with a homogenous distribution throughout the entire zone but with some densification in the central portion.

Corticalization was visible in all specimens at the end of the experiment. In the DBM group the callus area was slightly increased.

CT data are presented in table I for the total bone envelopes, indicating significant lower postoperative density values compared with the preoperative measurements The callus area was significantly different from the initial total bone size in all animals ($p < 0.01$), but there was a 278% increase in the DBM group which was significantly different from the OP-1 treated animals.

Both the OP-1 and the DBM group showed a lesser density in the lengthened area in comparison with the control animals, although the difference was not significant.

Histology

The findings were similar in all groups. The process of mineralization was clearly documented by the presence of mature bone trabeculae surrounded by osteoid fronts (fig 4). Corticalization was well underway in the distraction gap but with areas of incomplete bridging and irregular trabecular formation. Due to remodeling, a medullary canal filled with bone marrow was present. Neither additional periosteal bone formation nor extra induction of bone or cartilage were visualized in the treated groups.

DISCUSSION

The data from the present study do not show a significant difference between the three groups at 10 weeks postoperatively. According to quantitative measurements made by CT scan, there is an obvious lack of ossification at this stage, regardless of the treatment applied, as a maximum of 53% of normal density is reached, although radiographs show a well-filled distraction area. No statistical difference was found for the total amount of bone in the callus although absolute figures were higher for control than for OP-1 or DBM. DBM treatment resulted in almost threefold increase in the cross-sectional callus surface but with the lowest density of all three groups, indicating a large but weak callus.

Despite the well documented osteoinductive capacities of BMP's, both in research models and in clinical conditions, the administration of BMP-7 did not result in an additional bone formation in this specific setting (*1,7,17,26*). A limited study in patients with fresh tibial fractures treated with

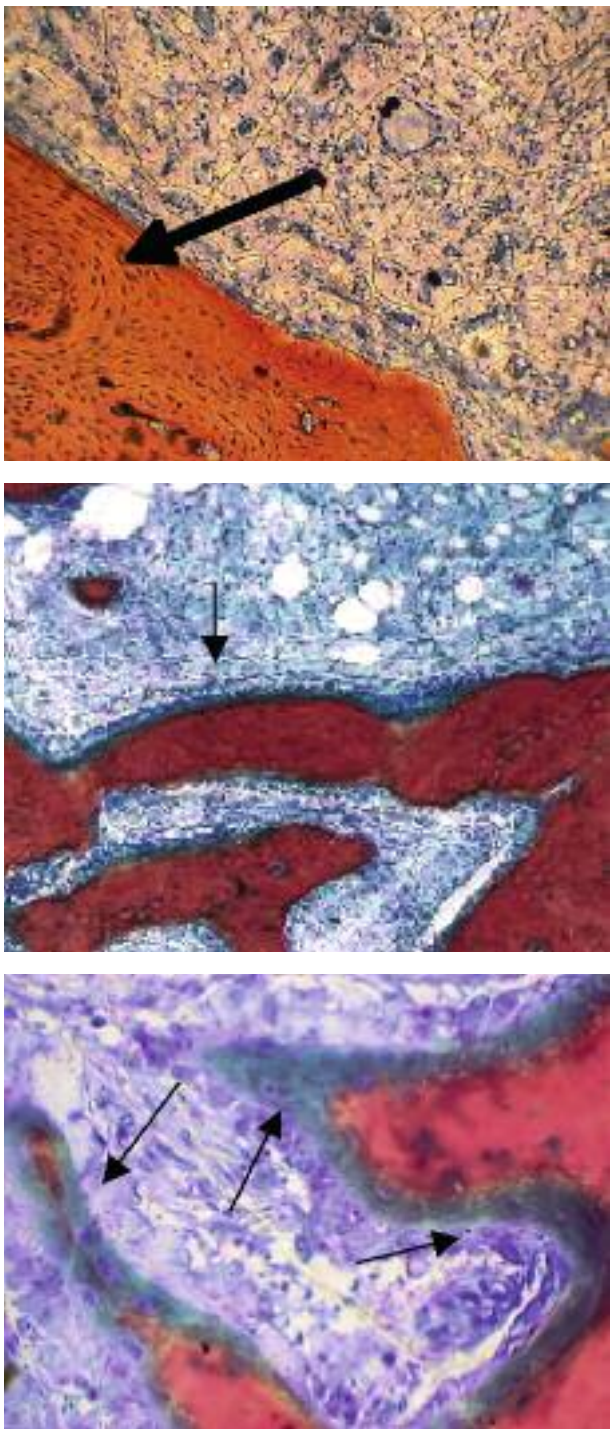

Fig. 4. — Detail of transverse histological specimen of an OP-1 treated animal showing mature bone trabeculae (1), clearly bordered with osteoid (2 and 3) as indicated by the arrows. Note the absence of cartilage due to intramembranous bone formation. (Gieson pichrofuchsine-Stevenel's blue coloring, magnification ×100).

OP-1 versus controls, which did not show a statistically significant difference in healing time, support these data to a certain extent (*20*). As in many other studies the used dose of 800 µg was supraphysiological, but it is only with this amount of protein that effects are seen in clinical situations. In laboratory conditions doses at which osteoblasts are influenced in their expression of growth factors and concomitant biochemical markers are more than 1000 fold less, rising the intriguing but unanswered question of dose related effects of OP-1 and other BMP's in general (*30*). In a rat femoral lengthening study, Mizumoto *et al* showed a significant effect with a dose of 20 µg (*21*). On the other hand Windhagen *et al* documented enhanced healing in a sheep tibial distraction model with administration of 8 mg BMP-2 at day 23 since the operation (*29*). Whether this 400 fold difference in concentration is simply species or time related or type of BMP specific remains unclear at present and is a challenge for further research.

In this study the exact time point of administration was chosen at the moment of operation. Due to the lack of knowledge of the specific temporal expression pattern of OP-1, there are no guidelines for the ideal amount of application. However, analogous to non-union treatment where OP-1 is applied immediately after debridement on the freshly bleeding bone surface, its use at the time of operation seems justified. This early application was also suggested by the findings of Hamdy *et al*, showing no effect with an injection at the end of the distraction period, due to the absence of BMP receptors in the consolidation phase (*10*).

As on one hand the bone healing cascade might be triggered by one single important growth factor, but as on the other hand bone repair is also the result of a concerted action of different growth factors, DBM seems to be a good source providing the necessary molecules for osseous regeneration, serving in the meantime as a conductive frame that acts as a supporting structure for host cells, but without offering mechanical stability (*9,28*). In this context DBM is advocated for the treatment of small cavities or as an adjuvant therapy for cancellous bone grafts when larger areas have to be reconstructed with graft reinforcement, such as a non-

union or a spinal fusion (*17*). Large scale experience with DBM in fresh fractures or osteotomies with or without distraction is not available. The present results, showing lesser densities than the control group do not suggest a strong inductive effect in this particular experiment. The larger callus area however indicates its conductive aspect. The considerable increase in the callus cross section that was observed can be considered as an enlarged area for potential bone formation. In contrast to OP-1 where one growth factor is administered in a well known dose, the quantification of the growth factors released by DBM is difficult. Different preparations might result in different effects on mineralization, and ideally *in vivo* assays with samples of the DBM used should accompany further experiments to have sufficient proof for its osteoinductivity.

Evaluation of bone development during different phases of callus distraction is routinely performed with standard radiographs, but they do not allow any quantification of callus formation and are prone to subjective evaluation. Dual Energy X-ray Absorptiometry (DEXA) has been reported, but this technique is not proposed for routine evaluation of limb lengthening, knowing its limited advantage over plain radiography in cases of small callus amounts (*2,18,19*). Moreover it is a projection technique and while the regenerate undergoes volumetric changes, spuriously aberrant mineralisation indices could have been produced.

Despite the potential error in area determination by the pQCT system, a significant positive relationship was documented between the compressive failure load of the callus and obtained callus size and densities in rat tibial fractures (*13*). Therefore CT can be considered to have a predictive value in estimating bone strength and thus to be a valuable tool for studying the influence of bone enhancing substances.

CONCLUSION

This study indicates that neither a one stage application of DBM nor the use of OP-1 at the time of surgery result in enhanced bone healing during bone distraction. Although an optimal environment is created in this well vascularized area of neohistogenesis an additional dose of growth factors does not raise an extra trigger. This extra stimulus might be strongly time and dose dependent and as long as the exact spatial and temporal expression pattern of these stimulating growth factors is not elucidated their *in vivo* applications will remain unpredictable. Local administration at the time of surgery failed to exceed the normal bone healing in control animals as measured by bone density. This indicates that it is difficult, at least in this specific setting, to compete with nature's normal healing pattern. The present results do not provide enough rationale for a routine use of DBM or OP-1 in clinical conditions of distraction osteogenesis. Notwithstanding these findings further analysis in relation to growth factor expression and osteoconductivity of DBM is essential and will be a subject of further research.

REFERENCES

1. **Burkus JK, Transfeldt EE, Kitchel SH, Watkins RG, Balderston RA.** Clinical and radiographic outcomes of anterior lumbar interbody fusion using recombinant human bone morphogenetic protein-2. *Spine* 2002 ; 27 : 2396-2408.
2. **Cattermole HC, Fordham JN, Muckle DS, Cunningham JL.** Dual energy X-ray absorptiometry as a measure of healing in fractures by intramedullary nailing. *J Orthop Trauma* 1996 ; 10 : 563-568.
3. **Cheng H, Jiang W, Phillips FM *et al.*** Osteogenic activity of the fourteen types of human bone morphogenetic proteins (BMPs). *J Bone Joint Surg* 2003 ; 85-A : 1544-1552.
4. **Cook SD, Baffes GC, Wolfe MW, Sampath TK, Rueger DC.** The effect of recombinant human osteogenic protein-1 on healing of large segmental bone defects. *J Bone Joint Surg* 1994 ; 76-A : 825-828.
5. **Edwards J, Diegmann M, Scarborough N.** Osteoinduction of human demineralized bone : characterization in a rat model. *Clin Orthop* 1998 ; 357 : 219-228.
6. **Friedlaender GE, Perry CR, Cole JD *et al.*** Osteogenic Protein-1 (Bone Morphogenetic Protein-7) in the treatment of tibial nonunions. *J Bone Joint Surg* 2001 ; 83-A (suppl 1, Pt 2) : 151-158.
7. **Geesink RG, Hoefnagels NH, Bulstra SK.** Osteogenic activity of OP-1 Bone Morphogenetic Protein (BMP-7) in a human fibular defect. *J Bone Joint Surg* 1999 ; 81-B : 710-718.
8. **Giltaij LR, Shimmin A, Friedlander GE.** Osteogenic protein-1 (OP-1) in the repair of bone defects and fractures

of long bones : clinical experience. In : Vukivecic S, Sampath T (eds). *Bone Morphogenetic Proteins* 2002, Birkhauser, Basel, pp 193-205.

9. **Greenwald S, Boden S, Goldberg V *et al*.** Bone graft substitutes : facts, fictions and applications. *J Bone Joint Surg* 2001 ; 83-A (suppl II) : 98-103.
10. **Hamdy RC, Amako M, Beckman L *et al*.** Effects of osteogenetic protein-1 on distraction osteogenesis in rabbits. *Bone* 2003 ; 33 : 248-255.
11. **Hughes TH, Maffulli N, Green V, Fixsen JA.** Imaging in bone lengthening. *Clin Orthop* 1994 ; 308 : 50-53.
12. **Ilizarov GA.** The tension stress effect on the genesis and growth of tissues. Part 2. The influence of rate and frequency of distraction. *Clin Orthop* 1998 ; 293 : 263-285.
13. **Jämsä T, Koivukangas A, Kippo K *et al.*** Comparison of radiographic and pQCT analysis of healing rat tibial fractures. *Calcif Tissue Int* 2000 ; 66 : 288-291.
14. **Jämsä T, Tuukkanen J, Jalovaara P.** Femoral neck strength of mouse in two loading configurations : method evaluation and fracture characteristics. *J Biomech* 1998 ; 31 : 723-729.
15. **Lammens J, Fabry G.** Reconstruction of bony defects using the Ilizarov bone transport techniques. A preliminary report. *Arch Orthop Trauma Surg* 1992 ; 111 : 70-72.
16. **Li G, Bouxsein ML, Lippen C *et al.*** Bone consolidation is enhanced by rhBMP-2 in a rabbit model of distraction osteogenesis. *J Orthop Res* 2002 ; 20 : 779-788.
17. **Ludwig SC, Boden SD.** Osteoinductive bone graft substitutes for spinal fusion : a basic science summary. *Orthop Clin North Am* 1999 ; 30 : 635-45.
18. **Maffulli N, Cheng JC, Sher A, Lam TP.** Dual Energy X-ray absorptiometry predicts bone formation in lower limb callotasis lengthening. *Ann R Coll Surg Engl* 1997 ; 79 : 250-256.
19. **Maffulli N, Cheng JC, Sher A, Ng BKW, Ng E.** Bone mineralization at the callotasis site after completion of lengthening. *Bone* 1999 ; 25 : 333-338.
20. **Maniscalco P, Gambera D, Bertone C *et al.*** Healing of fresh tibial fractures with OP-1. A preliminary report. *Acta Biomed Ateneo Parmenses* 2002 ; 73 : 27-33.
21. **Mizumoto Y, Moseley T, Drews M, Cooper VN 3rd, Reddi AH.** Acceleration of regenerate ossification during distraction osteogenesis with recombinant human bone morphogenetic protein-7. *J Bone Joint Surg* 2003 ; 85-A (Suppl 3) : 124-130.
22. **Nomura S, Takano-Yamamoto T.** Molecular events caused by mechanical stress in bone. *Matrix Biol* 2000 ; 19 : 91-96.
23. **Rabie AB, Wong RW, Hagg U.** Bone induction using autogenous bone mixed with demineralized bone matrices. *Aust Orthod J* 1999 ; 15 : 269-275.
24. **Reddi AH.** Bone Morphogenetic Proteins : from basic science to clinical applications. *J Bone Joint Surg* 2001 ; 83-A Suppl 1 Part 1 : 1-6.
25. **Reddi AH.** Role of Morphogenetic Proteins in skeletal engineering and regeneration. *Nat Biotechnology* 1998 ; 16 : 247-252.
26. **Salkeld SK, Patron L, Barrack RL, Cook SD.** The effect of Osteogenic Protein-1 on the healing of segmental bone defects treated with autograft or allograft bone. *J Bone Joint Surg* 2001 ; 83-A : 803-816.
27. **Schliephake H.** Bone growth factors in maxillofacial skeletal reconstruction. *Int J Oral Maxillofac Surg* 2002 ; 31 : 469-484.
28. **Tulie SM, Singh AD.** The osteoinductive property of decalcified bone matrix. An experimental study. *J Bone Joint Surg* 1978 ; 60-B : 116-123.
29. **Windhagen H, Witte F, Hurschler C *et al*.** Bone turnover during distraction osteogenesis in an experimental sheep model. *Arch Orthop Trauma Surg* 2002 ; 122 : 279-282.
30. **Yeh L, Unda R, Lee J.** Osteogenic Protein-1 differentially regulates the mRNA expression of Bone Morphogenetic Proteins and their receptors in primary cultures of osteoblasts. *J Cellular Physiology* 2000 ; 185 : 87-97.