JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 7 (1996) 63–67

Evaluation of hydroxyapatite powder coated with collagen as an injectable bone substitute: microscopic study in rabbit

B. FLAUTRE, G. PASQUIER*, M. C. BLARY, K. ANSELME, P. HARDOUIN
*Institut de recherche sur les Maladies du Squelette, Institut Calot, 62608 Berck sur Mer, France and *Centre Hospitalier Victor Provo, 59100 Roubaix, France*

New percutaneous bone filling techniques are beginning to be used by radiologists in bone tumour treatment because they are less agressive than surgery. The possibility of fostering the production of new bone using an injection method by catheter has prompted us to make pure hydroxyapatite (HA) coated with collagen (coll). The aim of this study was to assess three kinds of beads of 100–200 μm size by an injectable method in the distal part of femurs in rabbit. Three implanted groups were divided randomly: HA + COLL. group (*n*: 19); COLL. group (*n*: 10); HA group (*n*: 9). At intervals of 2, 4 and 8 weeks after surgery, bone ingrowth was evaluated on low magnification microradiographs (× 6.3) by an image analysing computer. This study has shown that bone formation induced by collagen in its isolated form is not as good as when associated with HA granules. At 4 weeks, a significant difference was observed between the three groups, with the lowest bone formation in the COLL. group. However, the amount of bone formation was small and needs to be improved.

1. Introduction

Orthopaedic surgeons are often confronted with bone loss either after trauma, infection or tumours. Many bone substitutes, synthetic or natural, have been proposed but their results are less reliable than with autologous cancellous bone graft, which remains the preferred method for healing bone defects. However, bone graft delays surgery time, increases blood loss and so may cause complications [1, 2]. Recently, the use of biomaterials to treat bone by percutaneous delivery has been proposed. Polymethylmethacrylate has been used to treat spine metastasis [3] and bone aneurysmal cyst [4]. Local injection of Ethybloc can give good results in the treatment of cysts [5]. These methods are interesting because they are less agressive than the usual surgery, but neither polymethylmethacrylate nor Ethibloc are bone substitutes and they are unable to replace bone grafts.

Hydroxyapatite (HA) is the main mineral component of bone, and synthetic porous HA is widely used as bone substitute because of its biocompatibility and its osteoconduction [6]. In contact with bone, HA develops a mechanically tight bond with it [7]. HA cannot be injected in its massive form. With HA powder, its size is compatible with injection but the biological properties of that powder must be improved by the addition of a vector. Collagen type I is the major structural protein in bone, comprising approximately 90% of its organic matrix. A viscous aspect obtained in collagen gel form can be used as a vector and can be injected by catheter [8, 9]. Therefore, granules of pure hydroxyapatite (HA) can be coated with collagen forming microspheres (HA + COLL.). The beads were made in order to obtain a bone substitute capable of injection and having two important components of bone.

A collagen gel may improve the dispersion of HA granules inside the finished product. The release of HA granules sustains a tight bond with the bone ingrowth inside the bone defect [10]. The evaluation of an injectable bone substitute needs a closed bone defect and a specific bone model has been proposed for this purpose [11].

2. Materials and methods

2.1. Materials

2.1.1. Biomaterials

2.1.1.1. HA powder. HA was precipitated in an aqueous medium by slow addition of diammonium phosphate [$(NH_4)_2(HPPO_4)$] solution containing NH_4OH to a boiling calcium nitrate [$Ca(NO_3)_2$] solution also containing NH_4OH. In addition, the suspension was refluxed before being filtered without washing. After filtration, the slurry was spray-dried at 1000 °C. The powder was composed of spheroidal granules of 47 μm mean size. X-ray diffraction (XRD) (Fig. 1) shows a pure HA, well crystallized with a density of 0.84 g/cm^3 and 50% porosity (furnished by C.E.A., France).

2.1.1.2. Collagen microspheres preparation. 1.6% of bovine atecollagen, unreticulated collagen type I and

0957–4530

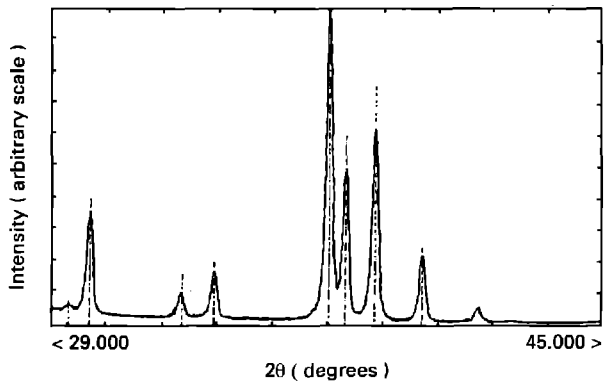


Figure 1 XRD of HAP "1000".

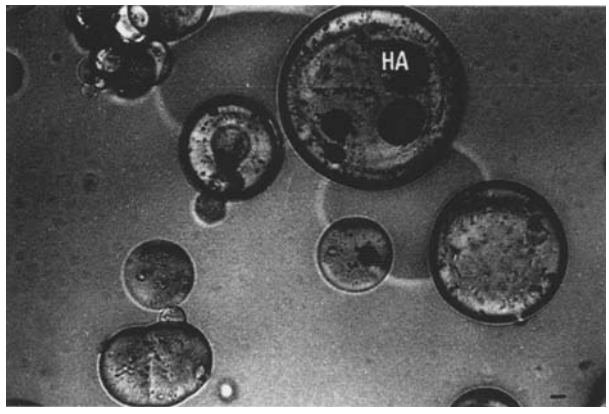


Figure 2 HA powder inside collagen in microbead form of 100–200 μm size. Bar = 33 μm.

0.8% of ovine chondroïtin-4-sulfate was mixed in a carbonate (0.6 M) buffered solution, pH 8. Then the solution was emulsified by stirring in an ethyl-2-hexyl cocoate bath (stearineries Dubois France). The individualized drops were reticulized by adding a terephtaloyle chlorure solution in ethyl-2-hexyl cocoate. During reticulation, the microspheres were recovered by centrifugation, washed several times in ethyl-2-hexyl cocoate baths, then in ethanol and finally in demineralized water. These microspheres with 100–200 μm mean size were scattered in a gel formed of 0.8% bovine atecollagen and 0.2% of ovine chondroïtin-4-sulfate. After lyophilization the mixture was sterilized by gamma radiation. The lyophilisate was scattered in sterile conditions in a buffered solution (0.1 M; pH 7.4) and then introduced in a system to be injected. The final product has the following composition: atecollagen/chondroïtin-4-sulfate: 11% by volume and phosphate buffer 0.1 M: 89% by volume.

2.1.1.3. Microspheres with HA. The procedure used was the same as described above. HA granules were incorporated at the rate of 5% in a chondroïtin-4-sulfate atecollagen solution. The mean size of the microbeads was 100–200 μm as illustrated in Fig. 2. The final product was atecollagen/chondroïtin-4-sulfate: 10.7% by volume and phosphate buffer 0.1 M: 66.7% by volume.

2.1.2. Animals

Nineteen white female New Zealand adult rabbits (controlled sanitary status), mean weight 3.820 kg. (range 3.6–5 kg) were included in the study. Rabbits were fed with standard rabbit chow pellets and tap water *ad libitum*.

2.2. Methods

2.2.1. Surgical procedure

After general anaesthesia by an injection of Ketamine (50 mg/kg) [Ketalar, Parke Davis, 92407 Courbevoie, France] in the auricular vein, two cavities per animal were made with a surgical approach at the lateral side in the distal femoral extremity, as recommended by Katthagen [12]. A cortical bone square of 8 mm side and 1 mm thickness was taken off and preserved to

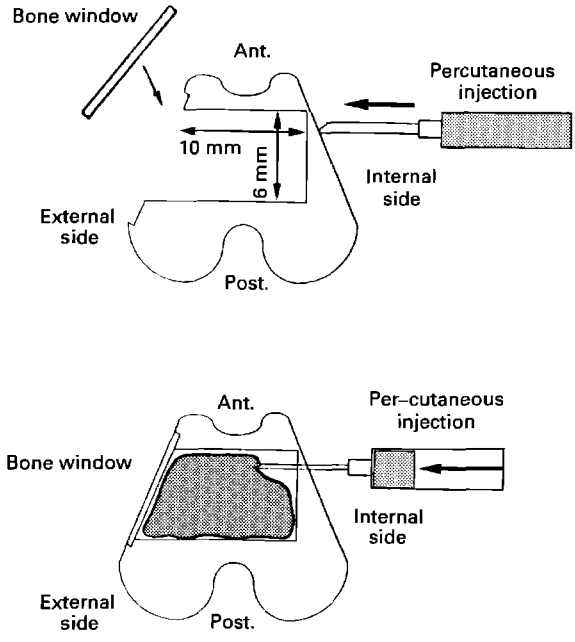


Figure 3 Percutaneous filling of the bone defect created in the distal femoral epiphysis of rabbit.

close the bone defect before or after filling it. The created defect was washed of bone debris and dried with a compress. The bone defect (6 mm diameter × 10 mm length) was made in a cancellous bone site of distal epiphysis (Fig. 3). An intramuscular needle was introduced into the defect by the lateral approach, punched through the internal cortex and crossed the internal skin. A large needle of 20 G size was threaded into the intramuscular needle which guided it into the defect percutaneously after shortening it to prevent the risk of pushing the cortical square during the injection. The injected volume in a cavity was 0.4 ml. The cavities were filled with either HA + COLL. (n = 19), COLL. (n = 10) or HA (n = 9).

After surgery, the animals were allowed to move freely in their cages without joint immobilization. The rabbits were sacrificed 2, 4 and 8 weeks after surgery. The distribution of cavities is given in Table I. Distal femurs were harvested, cleaned of soft tissues and fixed in 10% neutral buffered formalin.

TABLE I Distribution by time interval and kind of biomaterial used

Material	2 weeks	4 weeks	8 weeks	Total
HA + COLL.	7	6	6	19
HA	3	3	3	9
COLL.	4	3	3	10

HA: hydroxyapatite powder.
HA + COLL.: microbeads 100–200 μm size, HA coated with collagen COLL.
COLL.: microbeads 100–200 μm size, of collagen.

2.2.2. Microscopic study

Undecalcified bone preparation [13] was used for each specimen. Two 100 μm thick sagittal sections by sector (three sectors: external, medial, internal) were cut (Fig. 4) under cooling water with a sawing microtome Leitz 1600 (Microm, France). Microradiographs of these sections were performed on SO 343 Kodak (Cipec, France).

New bone ingrowth inside the defect was quantified with a semi-automatic method, on the microradiograph, by an image analysing computer VIDAS 3D (Kontron, France). The magnification used was × 6.3. Both first sections external and internal are excluded. The former because of the healing bone window interaction and the latter for its close relationship with the depth of the defect.

For each sector the evaluation comprised

- area of bone defect;
- area of new bone formation inside the bone defect (ratio area of new bone formation/total area of the bone defect);
- area of the residual HA inside the bone defect (ratio of residual HA/total area of the bone defect).

2.2.3. Statistical analysis

The results were expressed as the mean ± standard error of the mean. The three groups were compared using non-parametric Kruskall–Wallis's variance. When a significant difference was found a Dunn's test was applied. A minimum of $p < 0.05$ was required for a significant difference.

2.2.4. Qualitative examination

By transmitted light on undecalcified bone sections, 10 μm thick sections obtained by polishing the slices from 100 μm or 70 μm thick sections with an Exakt (Microm, France) under cooling water were stained using May Grünwald and Goldner colourations.

3. Results

Bone ingrowth related to bone defect is reported in Table II. Kruskall–Wallis's variance inter-groups shows a significant difference at 4 weeks without confirmation by Dunn's test. However, for the three kinds of beads, the new bone formation remains low, under 9%. With the HA microbeads group (Fig. 5a) because of excessive beads density inside, the defect cannot foster new bone formation. The HA–COLL. group shows a good pathway for the biological fluids where bony regeneration is identified (Fig. 5b). Therefore, the HA granules released after collagen degradation, sustain the new bone formation in increasing the bone strength (Fig. 7). But the delay in collagen degradation and its negative response (Fig. 5c) in increasing new bone formation in time (Fig. 6) decreases the efficiency of the HA + COLL. microbeads. Both groups HA + COLL. and HA microbeads have a good interface between existing bone as illustrated on microradiographs (Fig. 8a and 8b). Collagen microbeads have a general negative effect on bone formation.

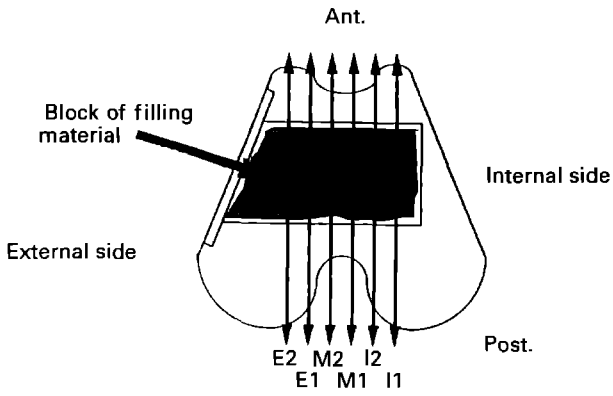


Figure 4 Distal femoral epiphysis of rabbit: transversal view. Topography of sagittal sections: E1: external section 1; E2: external section 2; M1: middle section 1; M2: middle section 2; I1: internal section 1; I2: internal section 2.

TABLE II New bone formation (%) inside the bone defect

Material	2 weeks	4 weeks[a]	8 weeks
HA	2.60[b] ± 1.40	4.50 ± 0.47	5.77 ± 2.83
HA + COLL.	3.98 ± 0.68	8.77 ± 3.28	8.84 ± 2.38
COLL.	4.02 ± 1.05	2.53 ± 0.44	6.66 ± 2.56

[a] significant difference between the three groups with Kruskall–Wallis's variance at 4 weeks (Dunn's test: NS)
[b] mean ± standard error mean

4. Discussion

In this study it is shown that HA powder coated with collagen can be injected; this material leads to better osteoconduction than HA powder or collagen alone, but the osteogenesis remains low. In a previous study [11] good reproducibility of defect size has been demonstrated. The mean surface area of the defect was 29.09 mm^2 ± 1.49 [11]. Therefore the size of the bone defect without bone substitute does not induce bone healing. The evolution of this closed defect allows the testing of many different bone filling materials, the use of which is ever growing in orthopaedic, orofacial and neuro surgery. In our HA + COLL. group the addition of HA to COLL. does not provide the good results obtained by some authors [12, 14]. In fact our HA or COLL. groups lead to weak new bone formation. We have used an experimental heated hydroxyapatite and not a sintered reference one [15], which could partially explain the different results. The fact

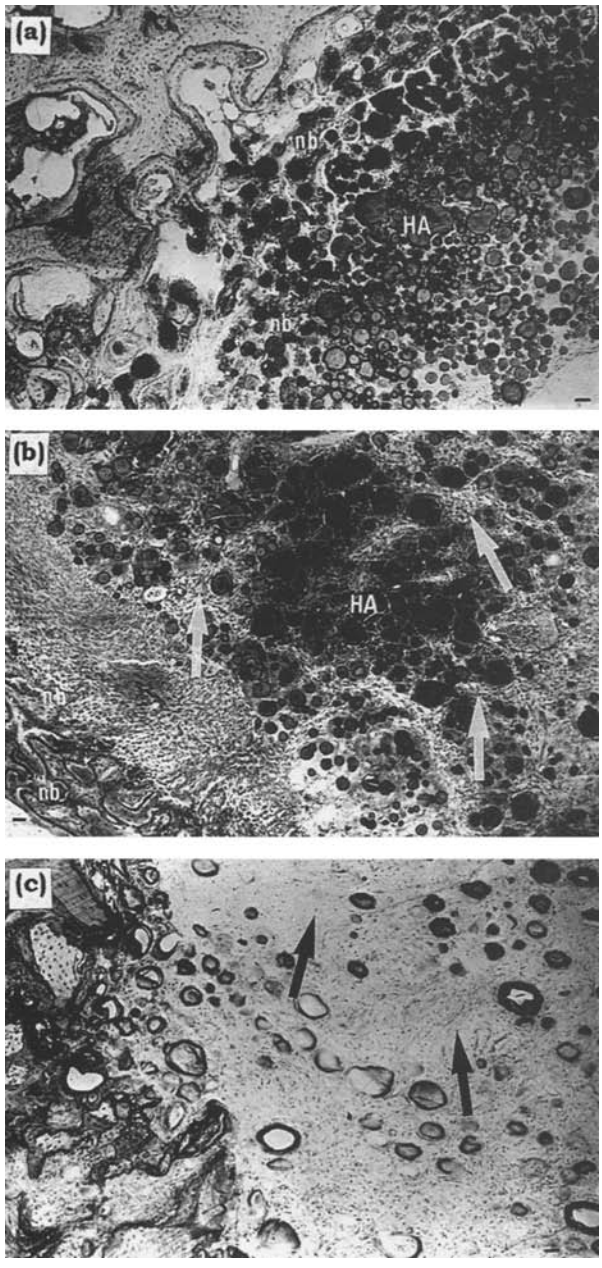


Figure 5 MGG colouration at 4 weeks (a) HA group: too great a density of HA granules prevents new bone formation (n.b.) inside the bone defect near the receiving bone (r.b.); (b) HA + COLL. group: microbeads show a good pathway for biological fluids (white arrows) compared to the HA group; (c) COLL. group: conjunctive tissue (black arrows) without new bone regeneration, only large pieces of collagen debris (d).

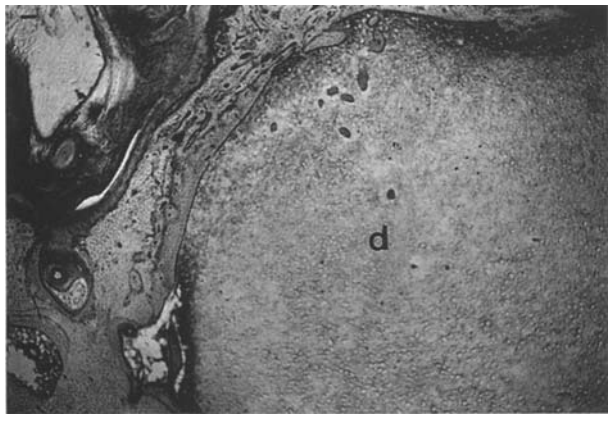
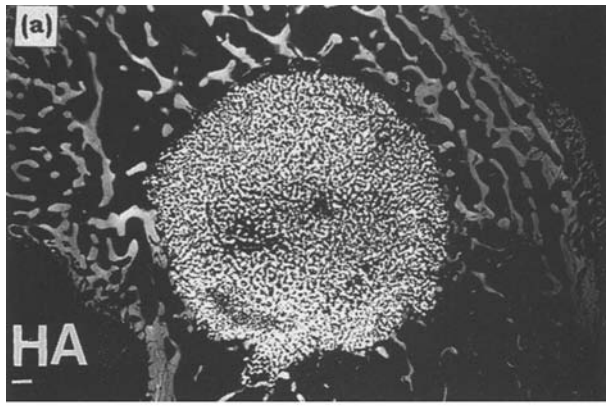


Figure 6 COLL. beads at 8 weeks: MGG colouration. No new bone regeneration (n.b.) in the defect (d). Bone formation (n.b.) is always close to the receiving bone (black head arrows). Bar = 18 μm.

that we have not used a collagen derived from a rodent species might contribute to its lower capacity to regenerate new bone formation because of the low degradation debris. In maxillo facial surgery, lyophilized collagen has accelerated the reconstruction of dentinal defect in human [15] and in created defects in rabbits [16]. Mittelmeier and Katthagen first imagined the scattering of hydroxyapatite inside a matrix to regenerate new bone with good results [17].

Figure 7 HAP + COLL. beads at 8 weeks: MGG colouration. HA granules sustain new bone formation to increase bone strength. Bar = 18 μm.



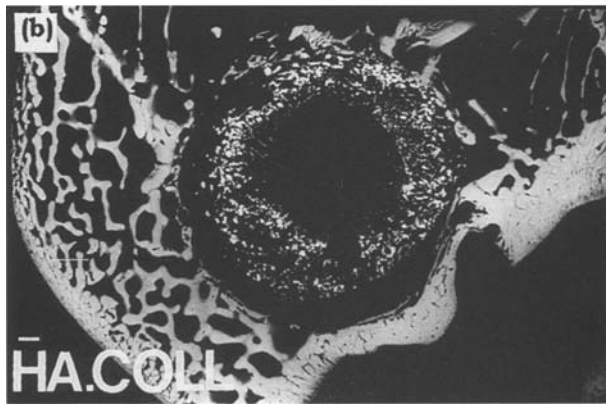


Figure 8 Microradiographs showing a good interface between receiving bone and HA or HA + COLL. microbeads (white head arrows). Bar = 45 μm.

5. Conclusions

Injectable bone substitute is an exciting concept with reference to percutaneous surgery, particularly in the association of HA and COLL. However, the new bone formation obtained with the biomaterials tested in this study appears insufficient and needs to be improved. Sintered HA and improved COLL. could be investigated.

Acknowledgements

The authors are grateful to BIOETICA 69007 Lyon for the preparation of HA + COLL. beads, doctor I. Orly for providing Fig. 2 and M. Court and R. Ranc, C.E.R.M, for providing the HA powder and Fig. 1.

References

1. M. B. COVENTRY and E. M. TAPPER, *J. Bone Jt Surg.* **A54** (1972) 83.
2. B. N. SUMMERS and S. M. EISENSTEIN, *ibid.* **B71** (1989) 677.
3. P. KAEMMERLEN, P. THIESSE, P. JONAS, J. DUQUESNEL, Y. BASCOULERGUE and C. LAPRAS, *N. Engl. J. Med.* **321** (1989) 121.
4. H. DERAMOND, R. DARRASSON and P. GALIBERT, *Rachis* **1** (1989) 143.
5. C. ADAMSBAUM, G. KALIFA, R. SERINGE and J. DUBOUSSET, *Skeletal Radiol.* **22** (1993) 317.
6. M. JARCHO, C. BOLEN, M. THOMAS, J. KAY and R. DOREMUS, *J. Mater. Sci.* **11** (1976) 2027.
7. M. JARCHO, J. KAY, K. GUMAER, R. DOREMUS and H. DRUBECK, *J. Bioengng* **1** (1977) 79.
8. L. S. COOPERMAN and D. MICHAELI, *J. Amer. Acad. Dermatol.* **10** (1984) 638.
9. A. M. KLIGMANN and R. A. ARMSTRONG, *J. Dermatol. Surg. Oncol.* **12** (1986) 351.
10. A. ROYER, J. C. VIGUIE, M. HEUGHEBAERT and J. C. HEUGHEBAERT, *J. Mater. Sci.* **4** (1993) 76.
11. G. PASQUIER, B. FLAUTRE, K. ANSELME, H. LECLET and P. HARDOUIN, Fourth World Biomaterials Congress, 24–28 April 1992, Berlin.
12. B. D. KATTHAGEN (Springer-Verlag, Berlin, 1987).
13. K. DONATH (Kulzer, Nordstedt, 1987) pp. 1–16.
14. D. R. MEHLISH, T. D. TAYLOR, D. G. LEIBOLD and R. HIATT, *J. Oral Maxillofac. Surg.* **45** (1987) 408.
15. B. KLAIBER, J. DUKER, U. JOOS and W. PERINO *Dtsch Zahnarztl* **35** (1980) 809.
16. U. JOOS, B. OCHS and P. E. RIES *Biomaterials* **1** (1980) 23.
17. H. MITTELMEIER and B. D. KATTHAGEN *Z. Orthop.* **121** (1983) 115.

Received 13 June
and accepted 30 December 1994