# Histological aspects in bone regeneration of an association with porous hydroxyapatite and bone marrow cells

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The osteogenic potential of an association of two kinds of hydroxyapatite (HA) porous ceramics with autologous bone marrow cells cultured with or without dexamethasone ($10^{-8}$M) addition in the culture medium and non-cultured rabbit marrow stromal stem cells (MSCs) was tested after 4 weeks of implantation in the dorsal muscles of spine in rabbit. A significantly higher number of rabbits with implants containing bone tissue inside pores were obtained with $10^7$ cells ml$^{-1}$ cultured treated with Dex. In the HA porous ceramic using naphtalen as porogen agent, the bone recolonization remains only at the periphery of implants and in the second row of pores, while in the HA porous ceramic using polymethacrylate (PMMA) microbeads as porogen agent, the bone recolonization is observed in the depth of implants. In the PMMA HA group, the Krüskal–Wallis variance analysis between the rabbits is significantly different with the percentage of number of occupied pores and occupied pores with bone tissue is different ($p < 0.05$).


## 1. Introduction

Many clinical situations in orthopaedic surgery require increasingly frequent use of bone substitutes. Hydroxyapatite (HA) and porous ceramics are widely used as bone substitutes because of their biocompatibility and their osteoconduction [1–3]. As a matter of fact, HA porous ceramics now have a growing place in bone pathology. New bone formation at the heart of the implant depends not only on the size and microstructure of the implant but also on the potential of cells in the grafted site. In this way, the osteogenic cells may be deficient in local grafted site. Results obtained with bone substitutes are currently less reliable than with autologous cancellous bone grafting which remains the preferred method for healing bone defects. Bone marrow stromal cells haved proved their ability to induce bone formation [4]. So the association of autologous bone marrow and HA porous ceramic might be a successful hybrid biomaterial for bone graft [5–7].

The aim of our study was to appraise bone tissue colonization inside such hybrid biomaterial according to the microarchitecture of the ceramic and to the type of stroma cells used. Two HA ceramics elaborated with two kinds of HA porogen agents and associated either with fresh autologous bone marrow or with cultivated bone marrow cells were used. Implantation was performed in the dorsal spine muscles in rabbit.

## 2. Materials

### 2.1. Biomaterials

Seventy-two discs of HA were used with $\sim 50\%$ of porosity, $\sim 200$–$300\,\mu$m mean pore size and a Ca/P ratio of $1.631 \pm 0.03$. Thirty-six discs were fabricated with naphtalen as HA porogen agent and 36 others with polymethacrylate beads (PMMA) HA as porogen agent [8] (Lamac, UVHC, Maubeuge, France). The disc size was 10 mm diameter and 2 mm height.

### 2.2. Sterilization

All the HA discs were sterilized by gamma irradiation at 25 kGy.

### 2.3. Animals

Twelve white female New Zealand rabbits $2.5 \pm 0.3$ kg (mean weight with sanitary controlled status) were used.

## 3. Methods

### 3.1. Bone marrow surgical harvesting

Under general anaesthesia, for each rabbit, bone marrow was harvested from one medullar midshaft of the femur through the trochanter.

### 3.2. Bone marrow cell culture

Bone marrow was resuspended in phosphate-buffered saline without calcium and magnesium (PBS −) by successive passages through 19, 22, 23 and 25 gauge syringe needles (Becton-Dickinson, Dublin, Ireland). Cells were resuspended in PBS − and counted using an automatic cell counter (Coultronics, France). After centrifugation, cells were resuspended in culture medium and plated in 25 $cm^2$ flasks and cultured with or without dexamethasone ($10^{-8}$M) in the culture medium. When primary cultures of osteoblastic precursors approached $\sim 90\%$ of confluence at about 3 weeks, the cells were harvested, detached with trypsin–ethylenediamine tetra-acetic acid (EDTA), counted and resuspended at $10^7$ or $10^8$/ml in serum-deprived α-MEM (minimum essential medium) medium after extensive rinsing with PBS − and verifying their phenotype three weeks later. Just before reimplantation, fresh bone marrow cells were harvested from the other medullar midshaft of the femur canal and prepared as described above, counted with $10^7$ or $10^8$ cells/ml in serum-deprived α-MEM medium before being impregnated in ceramics.

### 3.3. Ceramic impregnation

HA discs were impregnated either with $10^7$ or $10^8$ cells/ml of cultured and non-cultured cells under vacuum (−90 MPa) twice for 15-min periods. They were implanted randomly, as soon as possible, in the dorsal spine muscles of autologous rabbits for 4 weeks. Three samples were implanted on each side of the spine: non-cultured cells ± dexamethasone in suspension medium ($10^7$ or $10^8$ cells/ml), cutured cells ± dexamethasone in culture medium ($10^7$ cells/ml) and medium without cells. A control group was used with HA discs soaked in culture medium alone. Six samples of each type were implanted.

### 3.4. Microscopic evaluation

Before sacrificing the animals, a double label of oxytetracycline and alizarine was carried out. Three weeks after implantation, samples were harvested and the undecalcified method was used for bone preparation. Serial sections were made with an isomet saw (Krautkramer-Buehler, France) stuck on supports before being polished to 15 μm thick sections with Exakt system (Microm, France). Two white sections were used for ultraviolet light examination while two other sections were stained by May–Grünwald–Giemsa stain before being examined by optical microscopy. Histomorphometric evaluation was made with a point counting technique using an integrating eyepiece (100 points). The measured parameters were:

- Volume of pores occupied by cells or vessels or bone tissue in % related to implant section volume;
- Volume of pores occupied by bone tissue in % related to volume of total occupied pores;
- Volume of mineralized bone tissue in % related to volume of pores occupied by bone tissue.

### 3.5. Statistical analysis

The results are expressed by mean ± standard error mean (SEM). ANOVA of Krüskal–Wallis variance followed with Dunn's test was carried out between the rabbits, all implants mixed.

## 4. Results

A few days after the second harvesting of bone marrow cells, one rabbit was eliminated because of a fracture of the upper limb. In both groups of HA discs, a better bone tissue inside pores of ceramics was observed through using $10^7$ cells $ml^{-1}$ cultured with $10^{-8}$M dexamethasone added in culture medium rather than using bone autologous marrow cells (Table I). Then, in Dex-cultured PMMA–HA composite, the number of occupied pores was not significantly different whatever the rabbit and with all the implants mixed. But in histomorphometric evaluation the volume of occupied pores in percentage related to the total implant volume was significantly different between the rabbits ($p < 0.05$) and, therefore, the volume occupied by bone tissue ($p < 0.05$) (Table II). In addition, a better result was observed in the improved microstructure HA group. As a matter of fact, in the naphtalen HA group, the bone tissue was especially located in the opened pores at the periphery of implants and in the second row of pores (Figs 1a and 2a), the bone tissue *build-up* inside pores decreases with the depth of implants. In the PMMA–HA group, the bone build-up spreads to the heart of the ceramic discs and the amount of bone tissue build-up increases (Figs 1b and 2b). Whatever the HA group, two fluorescence appearances are seen, corresponding to primary and secondary bone tissue edification (Fig. 1a, b). The bone formation was also in relation to vessels and the presence of multinucleated cells were also close to the HA ceramic or in contact with new bone formation.

TABLE I Osteogenic potential of Dex-treated cultured cells in naphtalen HA and PMMA–HA implants after 4 weeks

| | Inoculated cells number/ml | Dexamethasone +/− (resuspension medium; non-cultured cells: cultured medium; cultured cells) | Number of implants containing bone tissue/total number of implants | |
|---|---|---|---|---|
| | | | Naphtalen HA | PMMA–HA |
| Non-cultured cells | $10^7$ | − | 1/6 | |
| | $10^7$ | + | 0/5 | 6/15 |
| | $10^8$ | − | 2/6 | |
| Cultured cells | $10^7$ | − | 2/6 | |
| | $10^7$ | + | 5/6 | 15/15 |
| Medium without cells | − | − | 0/5 | |

TABLE II Evaluation of total occupied pores and pores occupied by bone tissue in PMMA–HA hybrid material after 4 weeks in extra-bone site

| Rabbit | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| % of occupied pores by bone tissue | 40.053 ± 3.833 | 12.376 ± 8.713 | 11.647[a,b] ± 3.087 | 28.348 ± 8.949 | 37.808 ± 4.5 |
| % of total occupied pores | 60.438 ± 2.544 | 58.575 ± 4.742 | 48.357[c] ± 2.356 | 61.382 ± 2.094 | 68.173 ± 3.822 |

Results expressed as Mean ± SEM.
Statistics: Kruskal–Wallis test followed by Dunn's test: significant difference [a]*S*: 3 versus 2; [b]*S*: 3 versus 1; [c]*S*: 3 versus 5.

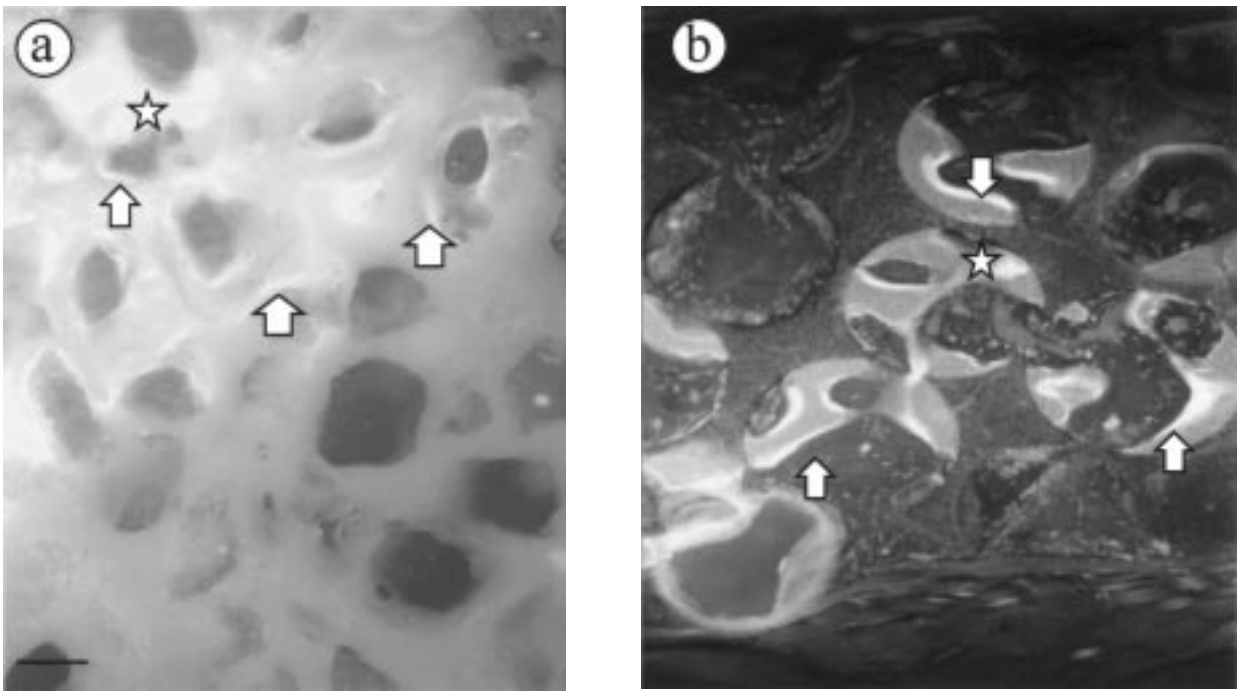


*Figure 1* Microscopic observation under ultraviolet light after 4 weeks of implantation (bar = 167 $\mu$m). (a) Naphtalen HA porous ceramic: the fluorescence labels are located at the periphery of implant and in the second row of pores. Linear labels (white arrows) and patches (white star) of primary bone edification. (b) PMMA–HA porous ceramic: linear fluorescence of lamellar bone in the depth of the implant (white arrows) and patches of primary bone (white star).

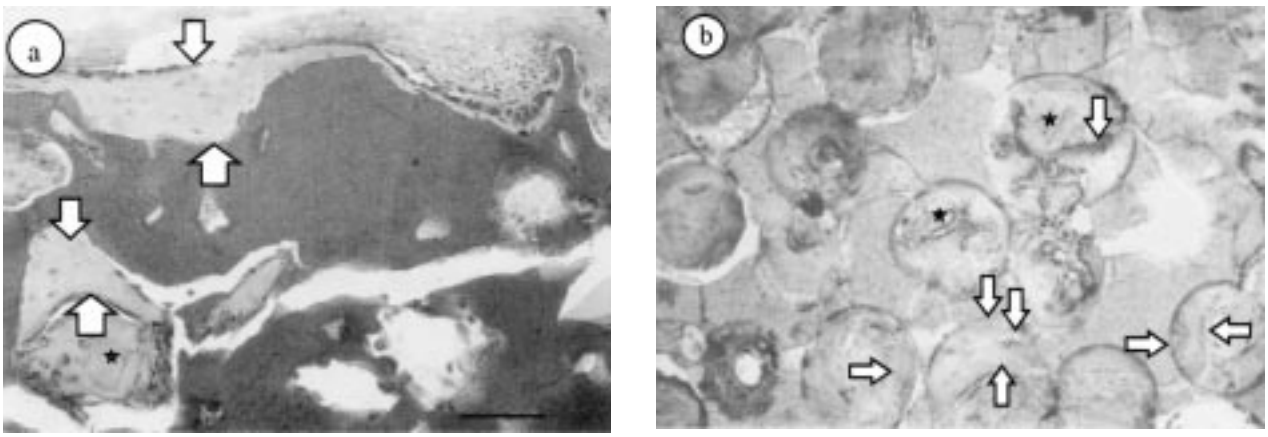


*Figure 2* Microscopic observation on stained section (May–Grünwald–Giemsa Staining; (bar = 143 $\mu$m). (a) Naphtalen HA porous ceramic: cells, vessels (white stars), but also cells able to build-up bone tissue limited at the periphery of the implant and in the second row of pores (white arrows). (b) PMMA–HA porous ceramic: cells, vessels (black star) and cells able to build-up bone tissue in the depth of the implant (white arrows).

## 5. Discussion

Our results, obtained with hybrid biomaterials, indicate that the inherent osteogenic ability of autologous bone stroma cells is stimulated by cell culture. This is in agreement with other authors [5–7]. Dexamethasone increases the *in vivo* osteogenic potential of cultured cells, which is consistent with previous results described by Yoshikawa *et al*. with rat marrow stromal stem cells (MSCs) [7]. Our results also raise the efficiency of an improved HA microstructure which confirms our previous *in vivo* and *in vitro* results from our experimental model in the distal part of the femur in rabbit [9, 10]. These data demonstrate the feasibility and the osteogenic capacity efficiency of this cultured cell/HA composite.

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*Received 13 May*
*and accepted 2 June 1999*