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Histological evaluation of xenogeneic bone treated by supercritical CO_2 implanted into sheep

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Untreated xenogeneic bone is known to be rejected when implanted into human or animal bone. It contains bone marrow tissue which is highly antigenic. To be used as an alternative to auto and allografts, this antigenic material must be entirely removed. Removal of this soft tissue contained in the bone structure was performed using a delipidation process with supercritical CO_2 followed by a deproteination with hydrogen peroxide or protease extraction. Such prepared materials were implanted into sheep bone for periods of 3 weeks, 2 months and 4 months. Bone whose organic matrix was destroyed by sintering, and untreated xenogeneic bone were used as controls. Qualitative histology and histomorphometry, measuring the percentage of the material in contact with newly formed, bone were performed on implant sections. This showed that the osseointegration of the supercritical CO_2-treated bone samples was equivalent to that of bone made anorganic by sintering while the untreated bone was embedded into an inflammatory tissue made up of macrophages, giant cells and plasmocytes.

1. Introduction

Bone allografts are used daily in human surgery. Although they seem well tolerated clinically, immunological reactions against osteochondral allografts have been detected in animals and humans [1–3]. Moreover, histological analysis of massive bone allografts has revealed that osseointegration was poor [4]; this was attributed to antigenic reaction against the graft [5].

Xenograft bone usually triggers a strong immunological reaction in the recipient organism [6]. However, partially deproteinated animal bone has been used in human surgery and has been well tolerated, showing good osseointegration [6]. "Kiel bone" is probably the most well-known xenogeneic bone graft available commercially. The bone is processed with hydrogen peroxide, treated with fat solvents, and dried with acetone. This material is very weakly antigenic [7].

The precise nature of the antigens responsible for inducing the allo or xeno reactivity has not been clearly determined. Bone contains a variety of potential antigens including cells: osteogenic, chondrogenic, fibrous, fatty, vascular, hematopoietic cells, and extracellular matrix: collagen, bone-specific proteins. The majority of the stimulation is supposed to be derived from cell surface glycoprotein antigens controlled by the major histocompatibility complex [8, 9].

One of the obstacles to bone cell and protein extraction is the presence of fat tissue in the medullary tissue limiting wettability and extraction by means of aqueous products. Furthermore, fat tissue contained in the bone pores has been proven to be antigenic [10] and to slow down the implant colonization. Fages *et al.* have recently described a new process for improving the organic matrix removal [11]. Supercritical fluid technology was applied on bone samples and appeared to be extremely efficient for removal of bone marrow components which are responsible for some immunological reactions. The use of carbon dioxide ensures a particularly safe and efficient delipidation operation since this fluid is known to be a good solvent for lipids [12]. Moreover, the unique properties of diffusivity (high diffusion coefficients), density (close to that of liquids) and viscosity (very low) of the supercritical fluids [13] imply that they are particularly adapted to the extraction of components entrapped in a solid microporous matrix, as is the case with bone. A specific deproteination step follows this deffating process. This step, which can be performed either with a chemical oxidizing agent or with a protease, is favoured since microporosity of bone tissue becomes much more accessible.

The aim of this paper is to determine the osseointegration properties (histologically defined for biomaterials as the amount of newly formed bone apposed on the implanted material) of such xenografts. Osseointegration of the cancellous bone material is the first step to incorporation by creeping substitution. Bone defects drilled in sheep femurs and tibias and filled with this material were used to evaluate its osseointegration.

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2. Materials and methods

2.1. Bones

Bovine femur condyles and femoral heads were used. Animals came from abattoirs with EEC approval and were killed for human consumption. After removal of all soft tissues, cylinders of 10 mm high and 6 mm diameter were cut using an "emporte-piece" tool in the cancellous part of the bone.

2.2. Supercritical extraction device

The pilot plant (Separex, Champigneulles, France) for continuous extraction and separation was located at the biochemical engineering department of the "Institut National des Sciences Appliquées" in Toulouse.

In this apparatus, cooled liquid carbon dioxide is pressurized by a metallic membrane pump (Dosapro-Milton Roy, USA) and then heated to the extraction temperature. The extraction vessel, with a volume of 200 ml, is followed by a series of four thermostatically controlled separation vessels, in which pressure is adjusted by needle valves. At the outflow of the fourth separator, CO_2 is liquified and recycled. At the bottom of each separator, a discontinuous recovery of the condensed phase is possible. A mass flow meter, based on the Coriolis effect, was used to determine mass flow rate.

Operating conditions were as previously published [11].

2.3. Proteic extraction

After supercritical CO_2 extraction, two different processes were used for partial extraction of bone proteins: hydrogen peroxide maceration at 40 °C for 12 h and protease maceration (Alcalase, Novo) at 50 °C for 24 h.

Two treatments (T1, T2) and two controls (C1, C2) were used for the implanted samples:

T1: bone cylinders extracted with supercritical carbon dioxide and hydrogen peroxide;

T2: bone cylinders extracted with supercritical carbon dioxide and protease (Alcalase, Novo);

C1: untreated bone cylinders;

C2: pyrolized bone cylinders heated at 800 °C to remove bone organic phase (anorganic samples).

All samples were sterilized by gamma-ray irradiation at a dose of 25 kGray (Conservatome, France)

2.4. Surgical procedure

Adult sheep were used for the experimentation and were stabled in the facilities of the surgical department of the National Veterinary School of Toulouse (France). After the sheep has been anesthetized, a lateral approach to the knee was made and a 6 mm hole was drilled either into femoral condyle or into tibial proximal epiphysis. The implant was aseptically introduced and the absence of micromovement was checked. Three sheep were implanted with each implant for each implantation period. After implantation periods of 3 weeks, 2 months and 4 months, animals were sacrificed using a pentobarbital injection and the femoral condyles and tibial epiphyses removed.

2.5. Histological analysis

The explants were fixed in a 5% buffered formaldehyde solution for 5 days and were dehydrated in increasing alcohol concentrations. They were embedded into polymethylmethacrylate (PMMA) and either 2 mm thick sections were made using a low-speed cooled diamond saw or 7 µm thin sections were made with a microtome for calcified tissue. Thick sections were fixed on the surface of a polystyrene block, and then ground on silicon carbide discs down to a 50 µm thickness. Then, they were stained with a fuscin-toluidine solution in a microwave oven at 2450 MHz and 200 W. Thin sections were stained either with hematoxylin-eosin or giemsa. They were observed with a Reichert Polyvar microscope.

2.6. Histomorphometry

The mean percentage of the implant perimeter in contact with new bone was measured using an image analysis device connected to a microscope (AES image, Toulouse, France). Five measurements on randomly chosen fields were made on each section at × 10 magnification.

2.7. SEM of the treated samples

After the extraction procedure, protease and hydrogen peroxide extracted samples were dehydrated in acetone and coated with gold-palladium. They were then observed using a Jeol JST-200 scanning electron microscope (SEM) at 25 kV.

2.8. Data processing

Mean values and standard deviations of the percentage of the implant perimeter in contact with bone were recorded and the time course of mean values was plotted.

3. Results

3.1. SEM of the sample before implantation

No organic remnants were visible in the pores of the treated bones. Osteocytes had disappeared from their lacunae. At high magnification the bone crystallites were visible at the material surface (Fig. 1).

3.2. Qualitative histology

3.2.1. 3-week implantation period

Control material (C1: untreated bone). No bone was apposed at the implant trabeculae surface. Inflammatory tissue made up of macrophages, giant cells and granulocytes was found in the periphery of the implant (Fig. 2). The implant pores were free of tissue ingrowth. They were filled with remnants of fatty tissue.

Anorganic bone (C2). All pores were invaded by a dense connective tissue which did not contain either macrophage or giant cells. The collagen fibres were parallel to the implant trabeculae except for some Sharpey's fibres inserted at the implant surface

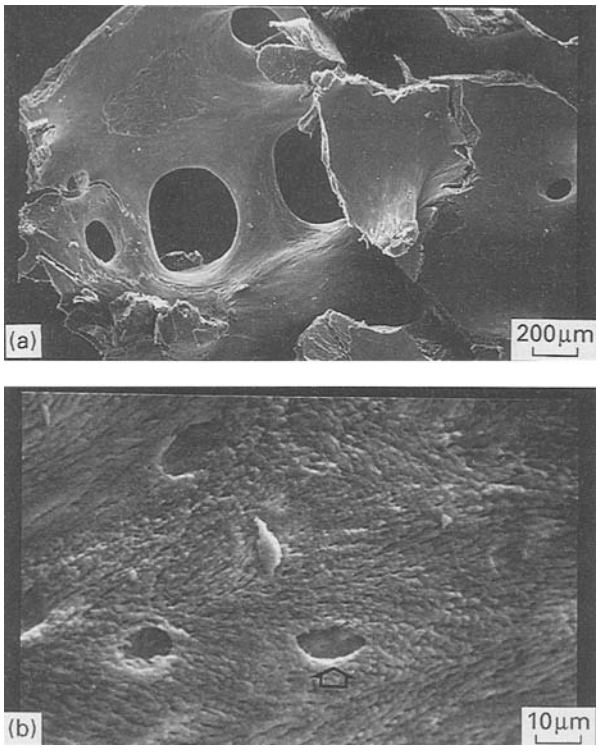


Figure 1 SEM microphotographs of hydrogen peroxide extracted bone (a) showing that the medullary tissue was removed by the treatment. (b) Higher magnification of the same material showing the mineral crystallites at the bone surface and the empty osteoplasts (op).

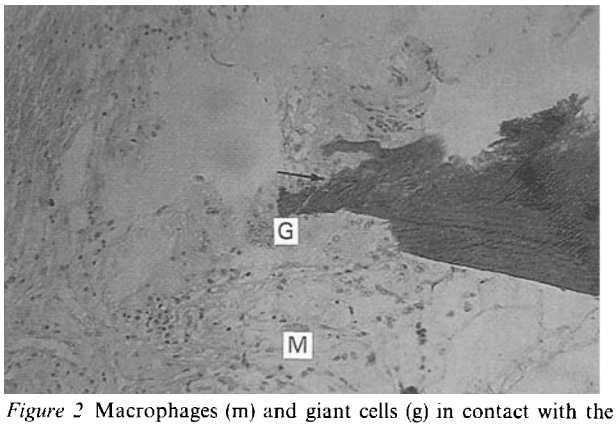


Figure 2 Macrophages (m) and giant cells (g) in contact with the untreated bone resorbing the material in focalized zones (↑)3 weeks after implantation. Fuscin-toluidine (× 450).

(Fig. 3). Some immature bone layers were adsorbed on some implant trabeculae of the external part of the cylinder.

Protease and hydrogen peroxide extracted material (*T1* and T2). The implant pores were filled with a dense connective tissue containing some hematopoietic zones. A thin layer of osteoid matrix was synthesized by osteoblasts on the surface of some trabeculae (Fig. 4). No histological difference was evidenced between the two kinds of implant (T1 and T2).

3.2.2. 2-month implantation period

Control material (*C1*). As for the 3-week implantation period, no bone tissue was found in contact with the

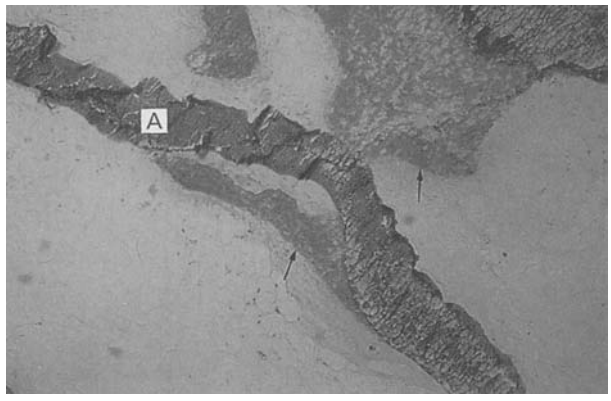


Figure 3 Anorganic bone (a) implanted for a 3-week period into a sheep bone (↑). The bone tissue is forming at the implant surface, some regions being still in contact with a loose connective tissue having invaded the implant pores by the early time of implantation. Fuscin-toluidin (× 50).

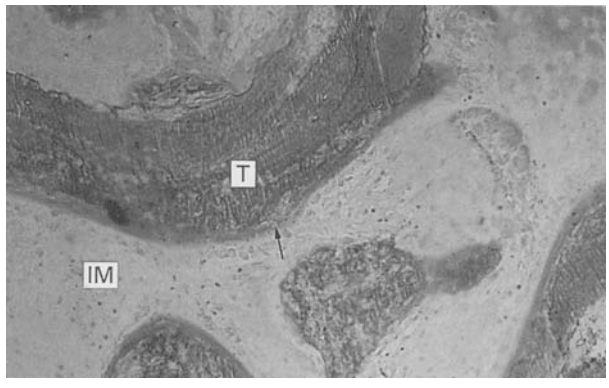


Figure 4 Three weeks after implantation of a hydrogen peroxide extracted sample, an osteoid layer (↑) was formed at the implant trabeculae contact (t). The rest of the pores were invaded by a dense connective tissue. (im) implanted bone. Fuscin-toluidine (× 50).

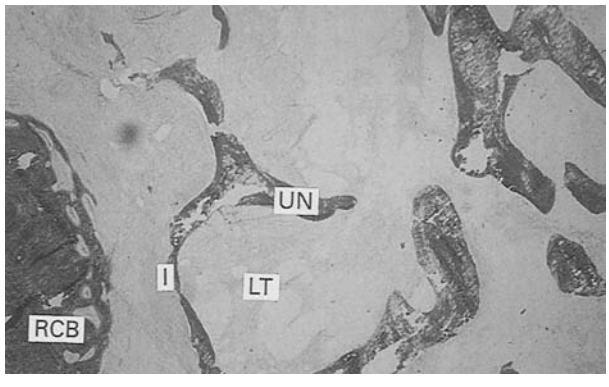


Figure 5 Two months after implantation, untreated bones (un) are separated from the receipient bone (rcb) by connective tissue. They are partially invaded by a loose connective tissue (lt) made of macrophages, giant cells and plasmocytes. Empty spaces and necrotic tissue remains inside the pores. Fuscin-toluidine (× 100).

implant. Implant pores were filled with a necrotic tissue appearing as a network formed by the juxtaposition of lipid vacuoles. At the implant periphery, a connective tissue composed mostly of macrophages and giant cells was seen. The inflammatory tissue ingrowth inside the pores is impaired by the necrotic tissue filling them (Figs 5 and 6).

Figure 6 Higher magnification showing the giant cells at the interface between the untreated bone and the loose connective tissue. Fuscin-toluidine (× 600).

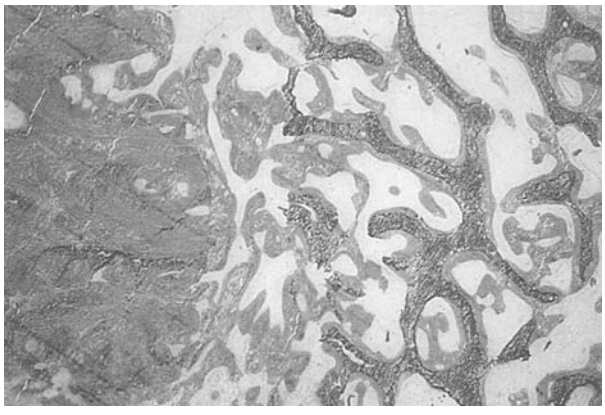


Figure 7 The majority of the surface of anorganic bone (black) was in contact with newly formed bone tissue layers (red) 2 months after implantation. Fuscin-toluidine (× 50).

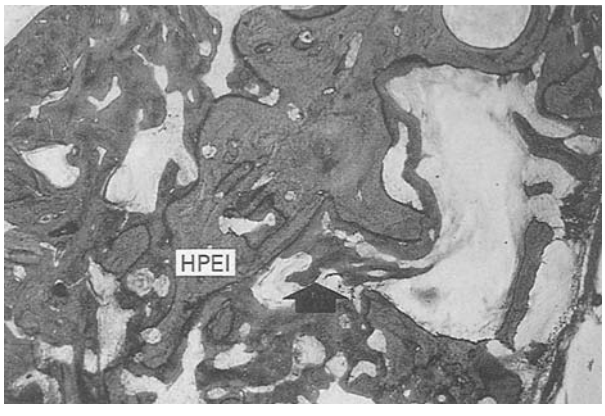
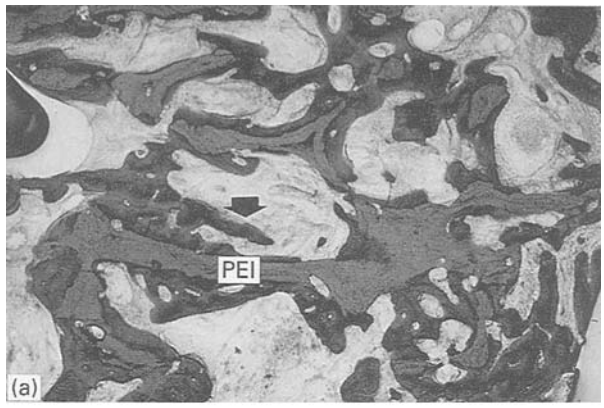


Figure 8 Hydrogen peroxide extracted implant (hpei) implanted for two months. Immature bone (*t*) had invaded the implant and covered most of its surface. Fuscin-toluidine (× 50).

Anorganic bone (*C2*). Immature bone layer was found on the whole implant perimeter. A few hematopoietic zones were found inside the pores (Fig. 7).

Hydrogen peroxide extracted implants (*T1*). Immature bone coated most of the implant surface (Fig. 8). Active osteoblasts were observed at the point of contact of osteoid tissue located on the immature bone surface. In implant regions which were not coated by immature bone, osteoclasts were found in Howship's lacunae resorbing the implant. As for the protease



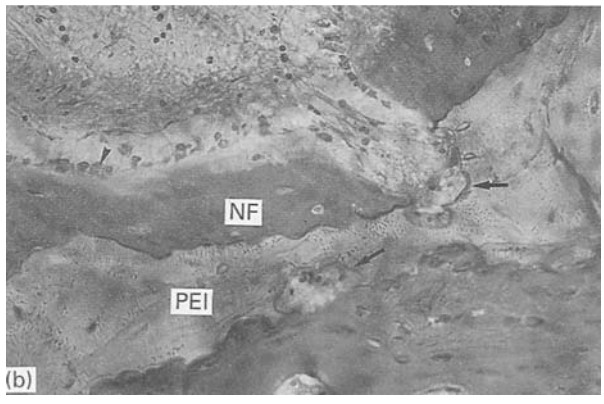


Figure 9 Protease extracted implant (pei) implanted for 2 months. (a) Immature bone (*t*) was formed at the surface of the implant trabeculae and coated most of this surface. Fuscin-toluidine (× 50). (b) Thick section showing the osseointegration of the implant by a creeping substitution process. Newly formed bone (nf) synthesized by active osteoblasts (▼) is adsorbed on the implant which is being resorbed (*t*). Fucsin-toluidin (× 550).

extracted implants, hematopoiesis fields were found inside the pores. Many lacunae were formed at the implant surface creating the first signs of implant fragmentation.

Protease extracted implants (*T2*). Osseointegration was high. Implant trabeculae were in contact with immature bone synthesized by active osteoblasts visible at the newly formed bone surface (Fig. 9). In the stromal tissue located inside the implant pores, hematopoiesis zones were identified. The implant showed some resorption marks on its surface. Osteoclasts were found in resorption lacunae dug in the implant.

3.2.3. 4-month implantation period

Control material (*C1*). No bone ingrowth was seen in the implant pores. One of the implants was almost totally resorbed leaving some bone fragments embedded into connective tissue (Fig. 10), which were surrounded by a few new bone tissue layers. No bone formation could be observed in the place of the resorbed material. As already observed for the other implantation periods, implants were surrounded by an inflammatory tissue made of macrophages and multinucleated cells.

Anorganic bone (*C2*). The cortical region of the implant was invaded by a dense immature bone,

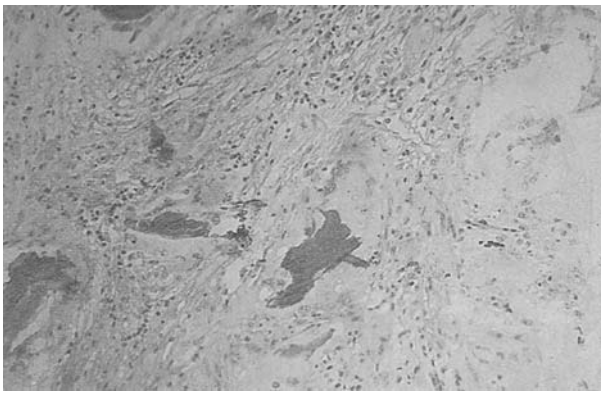


Figure 10 Four-month implanted untreated samples still embedded in an inflammatory tissue made of macrophages and giant cells when they have not been totally resorbed. Fucsin-toluidin (× 400).

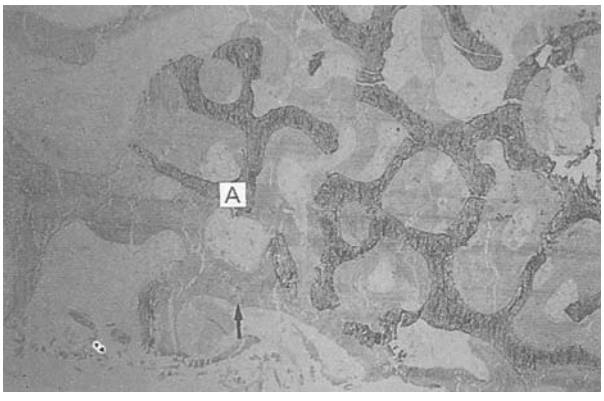


Figure 11 Anorganic bone (a) implanted for 4 months. The implant in its cortical region had been invaded by a dense bone tissue (↑) and is being fragmented. Fuscin-toluidine (× 50).

although the implant surface in the medullary cavity was coated by thin bone layers (Fig. 11). Resorption marks were evidenced on the implant surface and some macrophages containing calcium phosphate debris were shown.

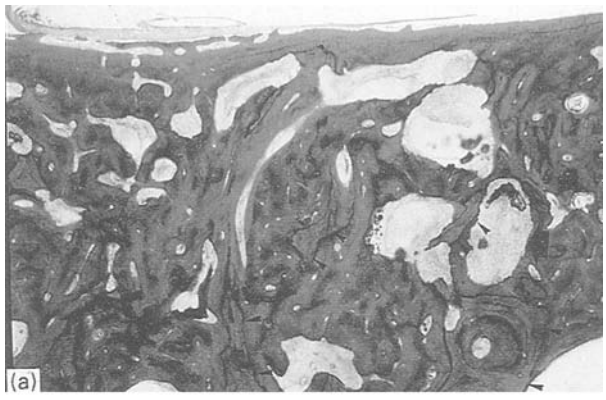
Hydrogen peroxide extracted implant (*T1*). On thin sections, the location of the implant was shown by the presence of a high remodelling process (Fig. 12). At the implant location, numerous resorption cavities suggested that the implant was going to be resorbed. Implant fragments could be identified inside the cortical bone on thick sections.

Protease extracted implants (*T2*). The observation of thick sections revealed that the implant was integrated inside newly formed bone. Implants were fractioned and the fragments were located inside cortical bone (Fig. 13). The implant trabeculae located inside bone marrow cavity were almost totally resorbed by osteoclasts. On thin sections, it is difficult to make a difference between newly formed bone and the fragments of the implant.

3.3. Histomorphometry (Fig. 14)

Control material (*C1*). No bone ingrowth nor apposition was seen until 4 months after implantation.

Anorganic bone (*C2*). 11.3% ± 8.9% (SD) of the implant surface was in contact with new bone after



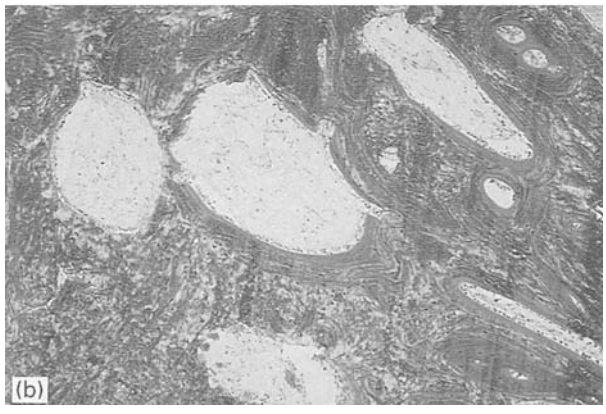


Figure 12 (a) Thick section of a protease extracted implant after a 4-month implantation period (⇑). The new bone was formed at the implant surface and some hematopoietic zones were visible in the bone marrow cavities. Fuscin-toluidine (× 50) . (b) Thin sections of the same regions as shown in (a). Thin sections make it difficult to see a difference between the implant and the high remodelled newly formed immature bone. Immature bone is characterized by an unorganized collagen matrix without formed osteons and large resorbing cavities with highly active osteoclasts. Fucsin-toluidin (× 250).

Figure 13 Thin section of hydrogen peroxide extracted implant (↑) after a 4-month implantation period showing that most of the implants have been resorbed and integrated in an immature high remodelling bone. Fuscin-toluidine (× 250).

a 3-week implantation period, more than 80% of the implant surface was in contact with new bone after 2 months (95.9% ± 4.1% at 2 months and 90.2% ± 10.1% after 4 months).

Hydrogen peroxide extracted implant (*T1*). 12% ± 8.1% of the implant surface was in contact with new bone after a 3-week implantation period, more than

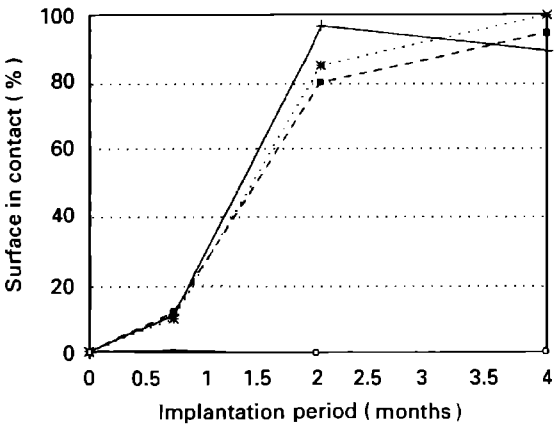


Figure 14 Percentage of the surface of the implant in contact with newly formed bone once implanted (-○- control (untreated); + anorganic; —×— protease extracted; -□- H_2O_2 extracted).

80% was in contact after 2 months (80.5% ± 20.2% at 2 months and 95% ± 5% at 4 months).

Protease extracted implant (T2). 10.3% ± 6.7% of the implant surface was in contact with bone after a 3-week implantation period, more than 85% was in contact after 2 months (85.1% ± 15.4% at 2 months and 96.1% ± 3.9% at 4 months).

In all cases except the untreated bone, at 3 weeks, the percentage of the external trabeculae surface in contact with bone was high, although bone tissue had not yet formed inside the implant.

4. Discussion and conclusions

This study shows that untreated xenogeneic bone is poorly tolerated by the recipient organism, as has frequently been described during the last 80 years [14, 15]. An inflammatory tissue was seen inside and around the untreated bone implants. Furthermore, a very strong osteolysis process could be shown for one implant during the 4-month implantation period. The delipidation and partial deproteination of xenogeneic bone suppressed the inflammatory reaction and made it possible to obtain good osseointegration. This new treatment improved the acceptance of the graft which was comparable to that of pyrolized bone. Anorganic bone, which is made solely of calcium phosphate, can be considered as a non-antigenic material. SEM of the treated bones showed that neither cells nor cell membrane remnants were left in the material, making unlikely an immunological reaction against the highly antigenic membrane proteins of bone marrow cells.

The delipidation by supercritical CO_2 associated with a partial deproteination seems to have an efficiency for bone ingrowth comparable to the delipidation performed using fat solvent maceration associated with deproteination using an oxidizing agent or proteic extraction techniques such as urea extraction [16]. It has been demonstrated that this kind of material is very weakly antigenic once implanted into humans, probably because bone marrow, which is the principal source of antigenic activity, has been removed [17]. Furthermore, the emptiness of the medullary cavities allows rapid connective tissue ingrowth inside the pores and the differentiation of osteoblasts from the stem cells found in the invading connective tissue.

However, structural proteins such as type I collagen are still present inside the material after the treatment and ensure that its mechanical properties remain unchanged.

The physical properties of supercritical fluids allow a total in-depth range penetration and therefore the delipidation of much larger bone volume than chemical treatment. Delipidation with fat solvents is much more difficult for such volumes.

Thermogravimetric analyses [11] have shown that the supercritical fluid treatments associated with protease or hydrogen peroxide extraction do not alter the collagen matrix and suggest a fragmentation of the collagen chains without modification of the mechanical properties. This fragmentation may decrease the type I collagen antigenicity which is already known to be low.

Even though serological probes of immunitary reaction were not sought, histological results show that this reaction is very low. Osseointegration is a key process for this material, acting as a framework for the bone healing tissue. During this short period of time (less than 4 months) the delipidation and the deproteination also improved the first step towards the incorporation of this material allowing a direct mineralization to occur at the implant contact.

These materials do not have osteoinductive properties due to the degradation or alteration of morphogenetic proteins during the partial deproteination stage. However, untreated allogeneic bone has never proved to be able to trigger an osteoinductive process.

References

1. I. YABLON, S. COOPERBAND and D. COVALL, *Clin. Orthop. Rel. Res.* **168** (1982) 243.
2. G. E. FRIEDLANDER, *ibid.* **174** (1983) 58.
3. M. C. HOROWITZ and G. E. FRIEDLANDER, *Orthop. Clin. North. Amer.* **18** (1987) 227.
4. W. F. ENNEKING and E. R. MINDELL, *J. Bone Joint Surg.* (Amer.) **73A** (1991) 1123.
5. V. M. GOLDBERG and S. STEVENSON, *Clin. Orthop. Rel. Res.* **225** (1987) 7.
6. R. SALAMA, *ibid.* **174** (1983) 113.
7. R. SALAMA and E. GAZIT, *J. Bone Joint Surg.* (Brit.) **60-B** (1978) 262.
8. V. M. GOLDBERG, A. POWELL and J. W. SCHAFFER, *J. Orthop. Res.* **3** (1985) 389.
9. P. F. HALLORAN, E. H. LEE and I. ZIV, *Transplantation* **27** (1979) 420.
10. P. ASPENBERG and K. THOREN, *Acta Orthop. Scand.* **61** (1990) 546.
11. J. FAGES, A. MARTY, C. DELGA, J. S. CONDORET, D. COMBES and P. FRAYSSINET, *Biomaterials* **15** (1994) 650.
12. R. P. de FILIPPI, *Chemistry and Industry* **19** (1982) 390.
13. J. F. P. HAMEL, R. C. WILLSON, J. C. ERICKSON and C. L. COONEY, *Biofutur* **51** (1986) 83.
14. H. BIRCHER, *Ann. Surg.* **6** (1987) 268.
15. M. J. KINGMA, *Arch. Chir. Neerl.* **12** (1960) 221.
16. D. CHAPPARD, C. FRESSONET, C. GENTY, M. F. BASLÉ and A. REBEL, *Biomaterials* **14** (1993) 507.
17. S. ESSES, P. HALLORAN and F. LANGER, *Trans. Orthop. Res. Soc.* **6** (1981) 78.

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