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Abstract

Different chemical or physical methods of bone processing have been developed to decrease the antigenicity of allogeneic bone which may delay or prevent graft integration. We have developed a method based on delipidation and deproteination of the bone with a supercritical fluid and hydrogen peroxide. Cylinders of cancellous allogeneic bone treated in this way were implanted for four weeks, four months or eight months in holes drilled in sheep condyles or tibial plateau. Histological sections were then processed and analysed qualitatively and quantitatively using an image analysis software coupled to a light microscope. Measurements were made of the trabecular bone surface (BS/TS), the relative osteoid surface (OS/BS), the active osteoid surface (OS/BS), active resorption surface (Oc.S/BS) and the relative surface of newly formed bone. After four weeks, the control cylinders (non-treated allogeneic bone) had been invaded by cellular tissue composed of lymphocytes and plasmocytes surrounding remnants of the donor bone marrow tissue. The processed cylinders showed osteoid apposition at the surface of their external trabeculae. The trabecular bone and osteoid surfaces were significantly higher in the processed bone sections than in the control bone sections. After four months, most of the control material had been osteolysed and replaced by connective tissue containing lymphocyte islets, while the processed materials showed a large amount of bone synthesized at the surface of implant trabeculae which appeared fragmented and disseminated within the newly formed bone. All the histomorphometric parameters measured were significantly different from those of the control.

By eight months, most of the control material had been totally osteolysed with very little bone ingrown in the implantation site. Only one control implant had been integrated. The processed cylinders were difficult to discern from the bone in which they were implanted. The parameters measured on the processed cylinders were significantly higher than those measured on the control sections.

In conclusion: the treatment applied to the bone enhanced allogeneic bone integration and could provide a new kind of tissue treatment for bone banking.

Keywords: Supercritical fluids; Bone allograft; Osteolysis; Immunity

1. Introduction

Bone allografts are used daily in human orthopaedic surgery although they present a risk of infection with certain viruses and bacteria [1]. They also show a risk of failure or non-integration which several authors have attributed to an immune response against the graft [2].

However, such bone material exhibits some advantages compared to synthetic materials which still make its use attractive. Its mechanical properties are very similar to those of the bone in which it is implanted and the presence of joint surfaces and muscle insertions are very useful in massive replacements.

There is thus a need to reduce both its immunogenicity and potential infectivity. The immunogenicity of the bone tissue is mainly due to the MHC proteins borne by the bone marrow cell membranes contained in the bone pores and medullary cavity [3].

Certain processes based on bone maceration in oxidative products, following defatting, can be used to eliminate the cells and cell debris from the processed bone [4]. These processes are not satisfactory due to the very poor

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wettability resulting from the high fat content of the bone and do not allow treatment of the bone volume that may be required for massive allografts in carcinologic surgery for example.

A physical process based on bone defatting with CO_2 in a supercritical state enables the lipids to be removed from a large bone volume which is then available for oxidative processing with aqueous solutions [5]. A supercritical fluid exhibits physical properties that are intermediate between those of a gas and fluid. Thus supercritical CO_2 ($SC.CO_2$) has the solvent properties of a fluid and the diffusivity of a gas which means that all the bone pores can be very quickly reached by the solvent. This process, furthermore, has shown a strong virucidal effect [6].

The aim of this experiment was to evaluate the histological integration of samples of cancellous bone allografts treated by this process and implanted within condyles or tibial plateau. This model was chosen because it had been already used [7] to evaluate the osseointegration of macroporous calcium phosphate ceramics which are taken as a reference material for osteoconductive material integration in cancellous bone. We treated cylinders of cancellous ovine bone. They were then implanted in the bones of non-donor sheep. The implanted zone was then subjected to histological analysis at various times.

2. Materials and methods

2.1. Material processing

Cylinders 5 mm in diameter and 10 mm in length were cut with a water jet in cancellous bone of vertebrae from a single sheep. These cylinders were either frozen at $-20^{\circ}C$ or treated with supercritical CO_2 and hydrogen peroxide. Briefly, the supercritical treatment was applied for 12 h as previously described [5], at 250 bars and at $50^{\circ}C$. A pilot plant (Separex, Champigneulles, France) for batch extraction and separation was used. Cooled liquid carbon dioxide was pressurised by a metallic membrane pump and heated to the extraction temperature ($50^{\circ}C$). The extraction vessel with a volume of 2.5 l was loaded with a titanium basket containing the bone samples to be treated. The extraction vessel was followed by a series of three thermostatically controlled separation vessels, in which the pressure was adjusted by needle valves. The CO_2 was liquefied and recycled at the outflow of the final separator. All the operations were remote controlled except for loading and unloading. Operating conditions were: CO_2 flow rate 2 kg h^{-1}, pressure 250 bars; extraction temperature $50^{\circ}C$; time 10 min g^{-1} of bone. The bone was then immersed in a 35% H_2O_2 solution at $40^{\circ}C$ for 24 h. It was then treated in NaOH solution (1 M) for 1 h at room temperature and buffered 15 min in NaH_2PO_4 (12 g l^{-1}), before being rinsed for 3 h in an ethanol solution (95%) followed by absolute ethanol for 2 h.

The characteristics of the processed bone have already been described [5]. Briefly, their composition was: mineral phase $63.5 \pm 0.5\%$; proteins $24.43 \pm 0.5\%$; lipids $0.5 \pm 0.01\%$.

Frozen samples were used as control implants. Both materials were sterilized by gamma irradiation (25 kGy).

2.2. Material implantation

The cylinders were implanted in holes 6 mm in diameter and 10 mm in length, drilled through a lateral approach, either in the external condyle or tibial plateau of different sheep to the donor. The implantation times were 1, 4 and 8 months. One processed implant and one control implant were implanted in four sheep for each period of time, the right leg receiving the control and the left the test sample. The sheep were killed by nembutal injection at the end of the implantation period.

2.3. Histological section processing

The condyles or tibial plateau were taken and fixed in a 4% formaldehyde solution in PBS (pH 7.6) for 48 h. The samples were then dehydrated in increasing ethanol solutions and embedded in PMMA. Thick sections (1 mm) were made using a low-speed diamond saw and ground to 50 μm thickness as previously described [8]. 7 μm thick sections were made with a Reichert Polycut type E microtome for calcified tissue.

Thick sections were stained with fucsin–toluidine after being etched for 10 min in a 1% formic acid solution, then 2 h in a 20% methanol solution. Thin sections were stained with fucsin–toluidine.

Thick sections were used because they allow easy differentiation of the new from the old bone without the need to label the ossification front.

2.4. Histological analysis

Different indices were determined in the implant sections. These included the trabecular bone surface (BS/TS), the relative osteoid surface (OS/BS), the active osteoid surface (OS/BS), the active resorption surface (Oc.S/BS) and the surface of newly formed bone (nfB/BS) (based on differences in stain affinity between the newly formed bone and the implanted bone). The data were obtained from a scanned area extending across sections of the implant from one edge to the other at the same level. Five sections made at a maximal distance of 1 mm from the cylinder center were measured.

The measures were obtained with an image analysis device using Graftek® software coupled to a light microscope.

2.5. Statistical analysis

Planned comparisons of means in ANOVA were performed for each period of time for each factor measured.

3. Results

3.1. Processed bone implants after four weeks

All the implants were macroscopically visible. The implant trabeculae were generally in contact with the bone in which they were implanted except for one implant that remained separated from the bone with no anastomosis. The external trabeculae of all implants were coated with osteoid tissue (Fig. 1). Active osteoblasts were apposed at the surface of the osteoid forming several cell layers in some places (Fig. 1).

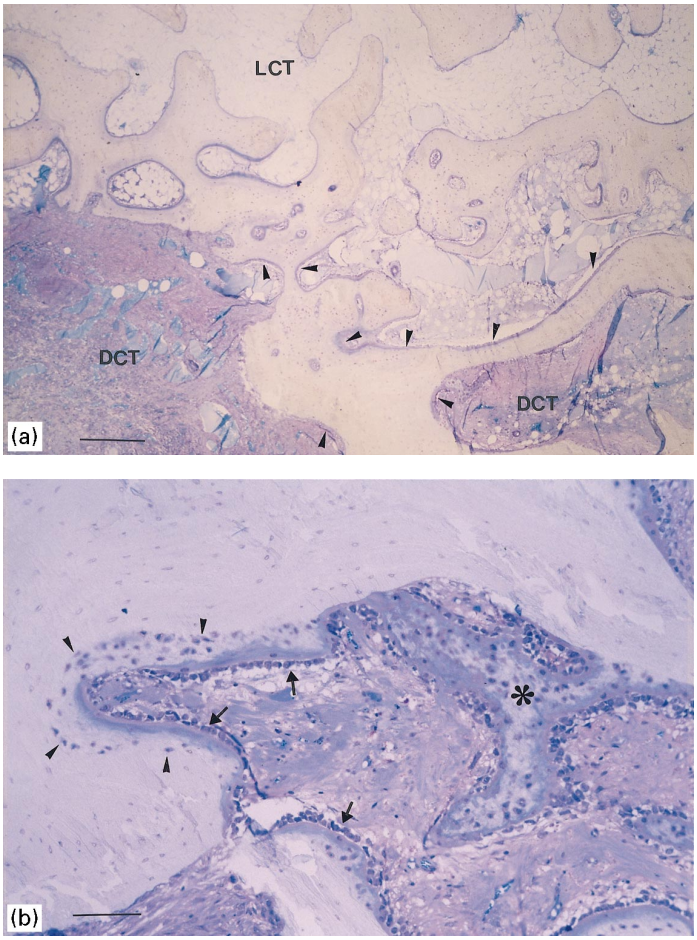


Fig. 1. (a) Microphotograph of the interface of a four-week-treated implanted cylinder with the recipient bone showing osteoid tissue (➨) coating the external trabeculae of the implant. The implant pores are filled with dense connective tissue (DCT) while the bone marrow cavity of the host bone is filled with loose connective tissue (LCT). Fucsin–toluidin. Bar: 300 μm. (b) Higher magnification of the same region showing the osteoid coated by an osteoblast multilayer (➡). Osteocytes (➨) are present in the living bone while they are absent in the implant. Immature bone (∗) is also formed due to the contact with the implant. Fucsin–toluidine, bar: 100 μm.

The pores of the implants were filled with a loose connective tissue containing islets of plasmocytes and an abundant vasculature. The pores of one implant contained some degranulating mastocytes. Resorption lacunae were found at the surface of one implant. They were filled with macrophages having phagocytosed calcium phosphate particles.

The trabecular bone and osteoid surfaces were significantly larger in the treated bone sections than in the control bone sections (Tables 1–4). The newly formed bone surface and the active resorption surface were not significantly different.

3.2. Control implants after four weeks

Macroscopically, the implants did not show any connection with the bone. They were, in all cases, separated from the bone by a thick cellular connective tissue. There was no osteoid on the external trabeculae or osteoblasts at the implant surface. There were signs of implant resorption.

The external pores were filled with a highly cellular tissue composed of lymphocytes and plasmocytes (Fig. 2a and b). The inner pores were filled with necrotic adipose tissue surrounded by hyaline tissue containing immune cells (Fig. 2c and d).

3.3. Processed implants after four months

The surface of the implant trabeculae was coated with a layer of newly formed bone in all implants.

Two implants showed limited areas of resorption without bone synthesis. One such area was located at the implant surface beneath the periosteum, and the other at the implant tip (Fig. 3a). Their cytological compositions were different from those of the integrated implant pores. One was characterized by the presence of mono- or multinuclear cells and active osteoclasts totally covering the edge of the cavity. The other was composed of a lympho-plasmocyte infiltrate limited by an osteoclast front.

In the integrated regions, the newly formed bone was differentiated from the bone implant by its staining affinity and by the absence of osteocytes in the implant structure. A highly active process of bone resorption had taken place at the implant surface. The implants were now composed of fragments of trabeculae dispersed within a matrix of newly formed bone (Fig. 3b–d). Most of the osteoid tissue coating the newly formed bone was in contact with active osteoblasts.

Table 1
Histomorphometric parameters measured on the implant sections expressed as the mean (μ) among implants and standard deviation (σx)

	Trabecular bone surface (BS/TS)	Osteoid surface (OS/BS)	Active osteoid surface (aOS/BS)	Newly formed bone surface (nfB/BS)	Active resorption surface (Oc/BS)
1 Month treated					
µm	29	11.8	9.2	1.5	2.35
σx	7.43	4.2	2.46	0.3	2.56
1 Month control					
µm	20.5	0	0	0	3.55
σx	1				14.5
4 Month treated					
µm	42.75	5.59	3.9	29.96	20.66
σx	6.25	4.19	1.9	10.54	14
4 Month control					
µm	0	0	0	0.5	0
σx				0.2	
8 Month treated					
µm	53.5	2	2	non-determined	1
σx	15.5	0.64	0.6		0.3
8 Month control					
µm	12.5	0	0	12.5	0
σx	4.33			4.33	

Table 2
Histogram of the mean relative trabecular bone surface on control and processed implant sections ± SD

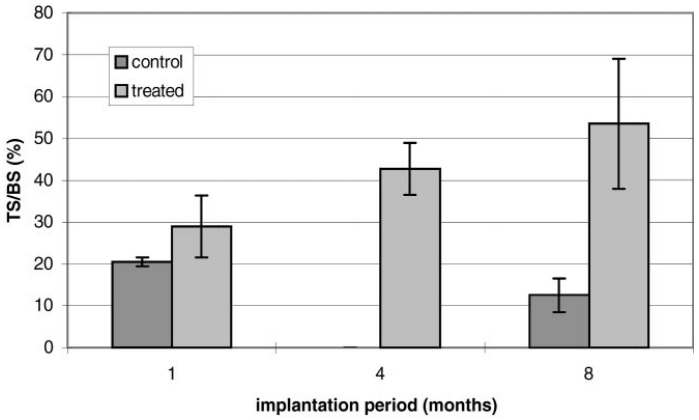
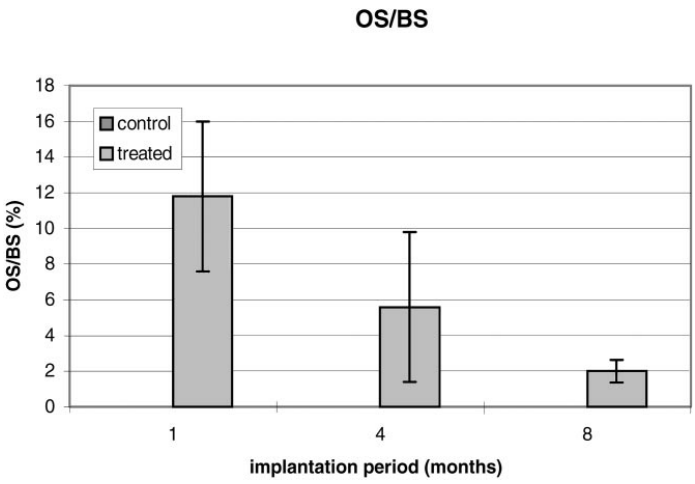


Table 3
Histogram of the mean relative osteoid surface in control and processed implant sections ± SD



All the histomorphometric parameters measured were significantly different from those of the control (Tables 1–4).

3.4. Control implants after four months

All the implants were degraded. A few trabecular fragments were found in a connective tissue which had invaded the implantation site (Fig. 4a). These fragments were found to be undergoing resorption by multinuclear cells. Certain fragments had been integrated at the periphery of the implantation site. The connective tissue that had replaced the samples contained islets of lymphocytes (Fig. 4b).

3.5. Processed implants after 8 months

Two kinds of implanted sites could be evidenced: sites which could not be differentiated from the implanted bone and those where the bone density and trabecular orientation showed that the implant had become integrated.

Very few implant fragments were identified.

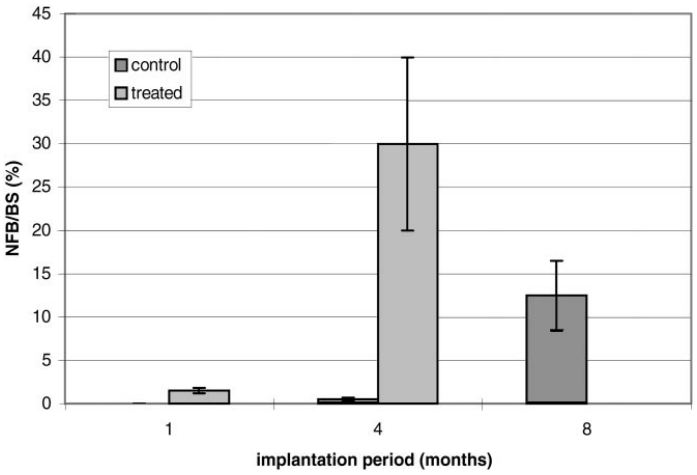
The parameters measured were significantly higher than those of the control sections (Tables 1–4).

3.6. Control implants after 8 months

One implant was partially integrated, with one-half showing total resorption. The other implants had been

either totally or partially destroyed. The holes which had previously contained these samples were filled with connective tissue containing small fragments of implant trabeculae.

Table 4
Histogram of the mean relative newly formed bone surface in control and processed implant sections ± SD



4. Discussion

All the allogeneic implants that had been subjected to treatment with supercritical CO_2 and oxidative agent had been integrated although some limited zones of osteolysis could be seen. Most of the implants that had not been treated had totally or partially disappeared and the resorbed parts had been replaced after four months by a fibrous tissue containing islets of lymphocytes or plasmocytes.

It is unclear why resorption of the untreated bone was so high. A histological study of morsellized allografts implanted in goat femurs had shown that most of the implanted bone was integrated and remodeled within 6–12 weeks although signs of a rapid lysis of the graft had also been noted [9, 10]. In our experiment, osseointegration of the untreated bone matrix was, in most cases, partial and limited to a thin rim of the samples. The non-integrated remainder of the implants had been lysed. The lymphocyte islets remaining around the connective tissue vessels suggest that an immunological reaction had taken place at the implantation site.

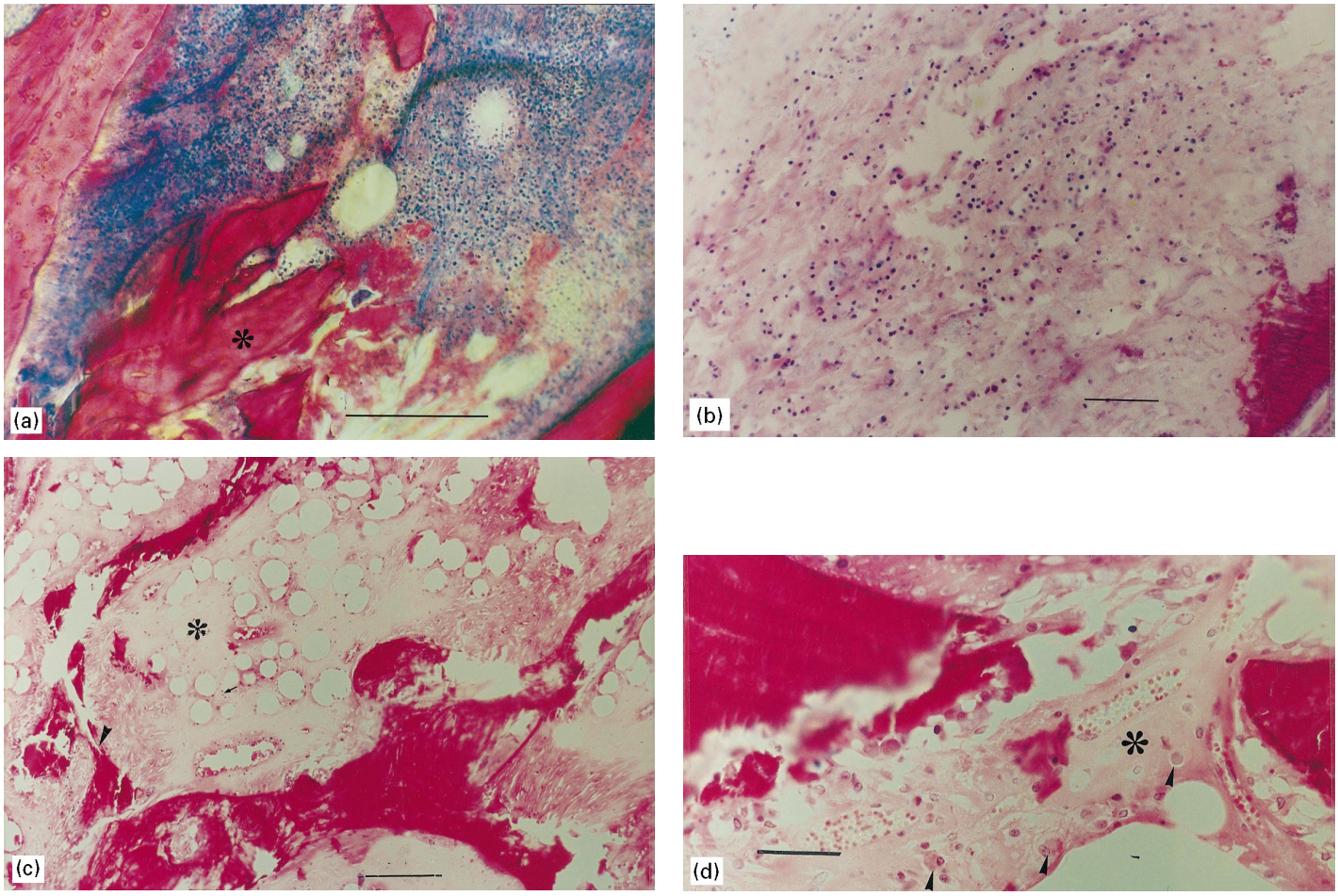


Fig. 2. (a) Micrograph of the ingrown tissue in the pores of the four 4 weeks implanted material (∗) composed of numerous lymphocytes and plasmocytes. Fucsin–toluidine, bar: 400 μm. (b) Micrograph of a thin section of the same implant. Implant trabeculae fragments are found in a lymphocytic infiltrate. Fucsin–toluidine, bar: 200 μm. (c) Micrograph of a thin section of the hyaline tissue (∗) contained in the inner pores of a four-week-implanted control material. Plasmocytes (➡) are disseminated in this tissue while the bone trabeculae are being degraded (➡). Fucsin–toluidine, bar: 400 μm. (d) Same section at higher magnification. Plasmocytes (➡) are clearly visible in the hyaline matrix (∗) between the degraded bone trabeculae of the implant. Fucsin–toluidine, bar: 100 μm.

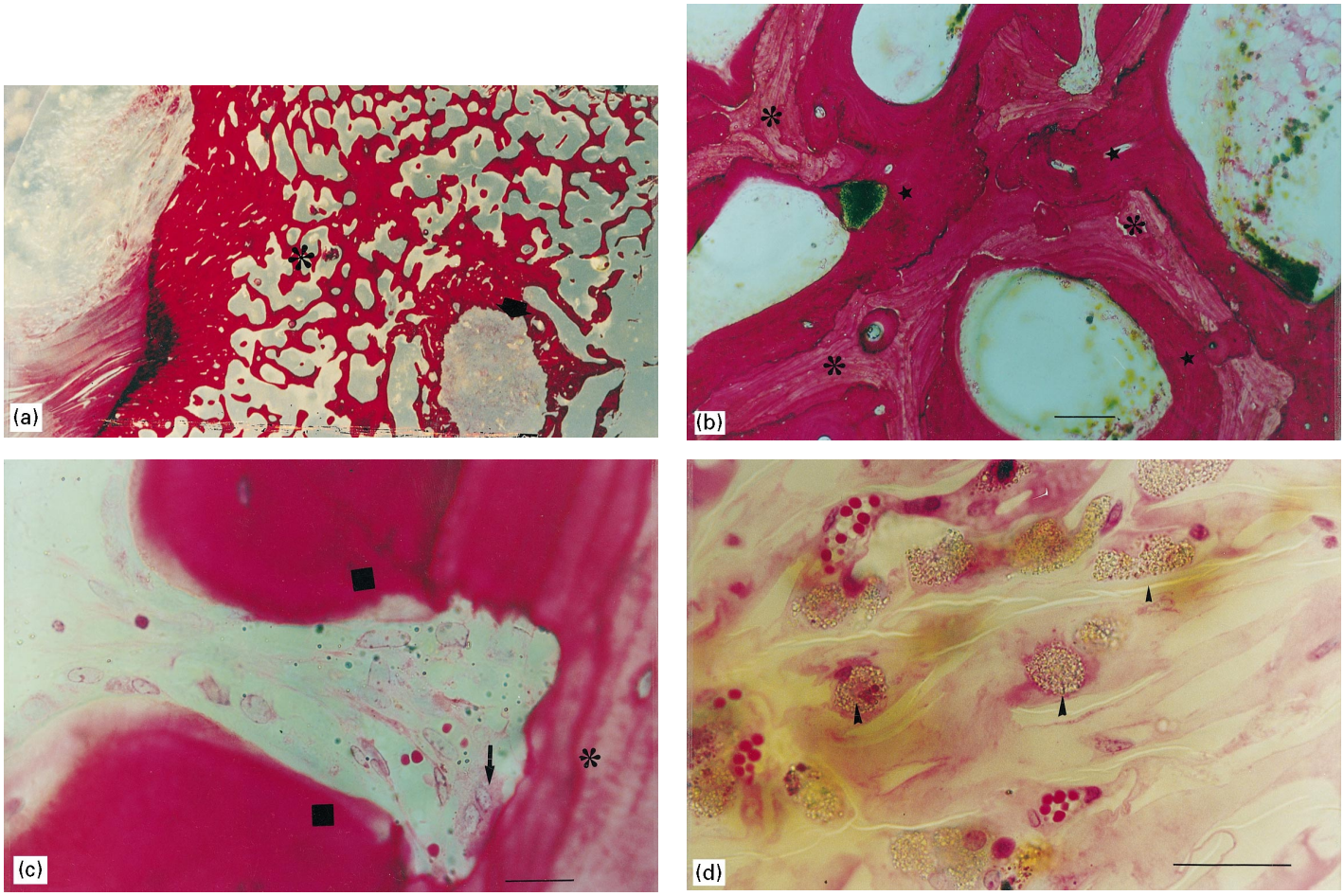


Fig. 3. (a) Photograph of a section of a four-months-implanted treated implant showing the external part of the implant perfectly integrated (∗) and an osteolytic zone at the tip (➡). Bar: 10 mm. (b) Micrograph of a processed sample implanted for four months. The implant trabeculae appear as fragment (∗) disseminated in the newly formed bone matrix (★) suggesting a process of creeping substitution. Fucsin–toluidine, bar: 100 μm. (c) Remodelling process taking place at the surface of a four-month-processed implant showing an osteoclast (➡) resorbing the implant trabeculae (∗) after the newly formed bone (■) deposited at their surface. Fucsin–toluidine, bar: 50 μm. (d) Fragments (➡) of the implanted processed bone phagocytosed by the histiocytes found in the pores of a four-months-treated implant. Fucsin–toluidine, bar: 50 μm.

The small amount of untreated bone that had been integrated had been protected from total degradation. It seemed that cleansing by macrophages of the necrotic tissue remaining in the bone pores of the untreated material had also affected the bone trabeculae. It has been shown that activated cells of the immune system and their soluble products can induce osteoclastic bone resorption [11]. In our experiment, resorption of the non-treated material was secondary to invasion of the material pores by immune cells and it is therefore suggested that such osteolysis was a consequence of immune system activation.

These results suggest that most of the samples had been implanted across a strong histocompatibility barrier. As the donor and receiver animals belonged to two homogeneous but genetically distinct groups of animals, this could have created a bias responsible for the high rejection rate.

It is very difficult to extrapolate the behaviour of small cancellous bone samples to that of massive allografts. Moreover, the control material had not been chosen to model the clinical situation. However, at the trabecular level, interesting information could be obtained about the behaviour of the healing tissue in relation to the implant. Due to its unique diffusion property, supercritical fluid can be adapted to treat a large bone volume. The efficient volume of delipidation can be improved by increasing the time spent within the $SC.CO_2$. The efficient bone volume for bone marrow deproteination can, as has previously been described be greatly enhanced by dissolution of protease in the $SC.CO_2$ [12]. It is therefore likely that the treatment of large bone volumes by SCF technology could facilitate their subsequent integration.

Identical implantations were made with inorganic bone allografts obtained by sintering cancellous bone cylinders at more than 400°C. A process of creeping substitution was demonstrated at the contact of these samples which were rapidly integrated within a few weeks and resorbed within eight–nine months [7]. No material osteolysis was ever noted.

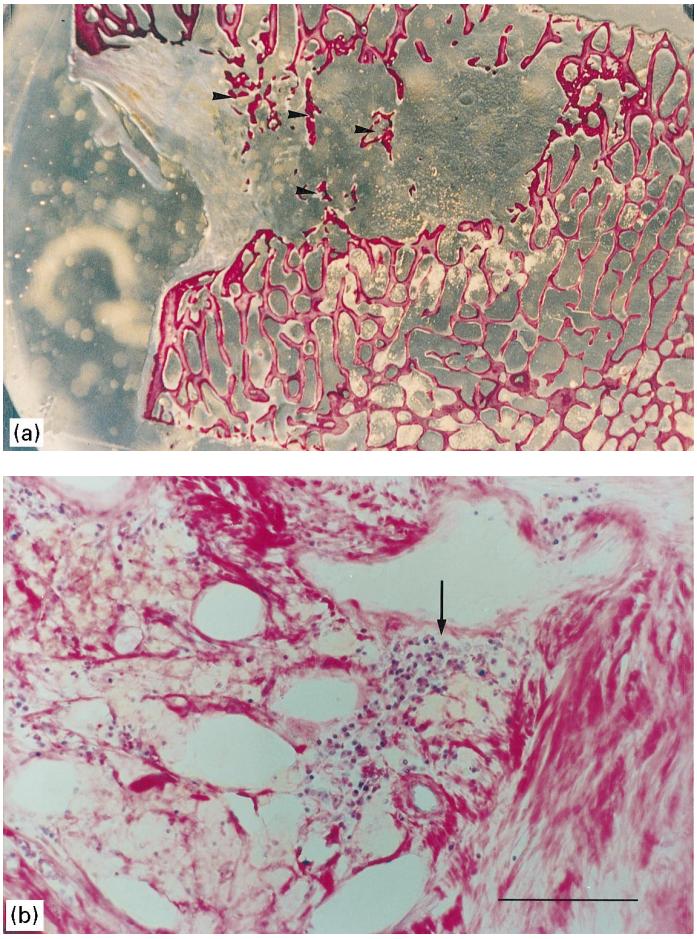


Fig. 4. (a) Photograph of a four-month-implanted control sample showing an implant osteolysis leaving some fragments of degraded trabeculae in a connective tissue which had invaded the site (➨). Bar: 10 mm. (b) Micrograph of the connective tissue which had invaded the site of a control sample after it had been osteolysed. Islets of lymphocytes (➡)are disseminated within the connective tissue. Fucsintoluidine, bar: 100 μm.

5. Conclusions

Removal of the bone marrow tissue from bone facilitates the integration of small allogeneic bone samples. As supercritical CO_2 treatment allows the removal of bone marrow from large pieces of bone, it can be extrapolated that the subsequent integration of such materials will be enhanced.

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