Bone allografts and supercritical processing: effects on osteointegration and viral safety

Jacques Fages, E Jean, P Frayssinet, D Mathon, B Poirier, A Autefage, D Larzul

▶ To cite this version:

Jacques Fages, E Jean, P Frayssinet, D Mathon, B Poirier, et al.. Bone allografts and supercritical processing: effects on osteointegration and viral safety. Journal of Supercritical Fluids, 1998, 13 (1-3), pp.351-356. 10.1016/S0896-8446(98)00071-0 . hal-01697288

HAL Id: hal-01697288
https://hal.science/hal-01697288

Submitted on 21 Mar 2018

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Bone allografts and supercritical processing: effects on osteointegration and viral safety

Jacques Fages [a,*], Eliane Jean [b], Patrick Frayssinet [b,c], Didier Mathon [d], Béatrice Poirier [e], André Autefage [d], Daniel Larzul [e]

[a] *Ecole des Mines 81013, Albi, cedex 09, France*
[b] *Bioland, 31100 Toulouse, France*
[c] *Lab tissu osseux et pathologies ostéoarticulaires, Université P Sabatier, 31059 Toulouse, France*
[d] *Ecole Nationale vétérinaire, 31079 Toulouse, France*
[e] *Texcell, Institut Pasteur, 75015 Paris, France*

Abstract

A new bone tissue process using supercritical carbon dioxide extraction was evaluated for viral inactivation and the allografts produced by this process were tested in an in vivo implantation experiment. Four viruses, human immunodeficiency virus type I (HIV-1), Sindbis virus, Polio Sabin type I virus and Pseudorabies virus (PRV) were assayed. Four processing stages, supercritical CO_2, hydrogen peroxide, sodium hydroxide and ethanol treatments were also tested. The efficiency of the process was assessed in terms of reduction factors which are the $\log_{10}$ of the ratio of the virus load before and after the stage to be evaluated. The cumulated reduction factors were the following: >18.2 for Sindbis virus, >24.4 for Poliovirus, >17.6 for PRV and >14.2 for HIV-1. Such allografts processed in this way were implanted into sheep leading to a much faster osseointegration in comparison with non-treated allografts. The combination of better graft incorporation and viral safety suggest that this process could become a new way for processing bank bones, alternatively or additionally, to the procedures presently used.

Keywords: Bone allografts; Viral safety; Supercritical CO_2; HIV1 virus; Osseointegration

1. Introduction

Because of the development of modern orthopaedic surgical techniques, there are increasing demands for bone allografts throughout the world. To improve availability, efficiency and infectious disease safety of these grafts, bone banking procedures were increased tremendously over the past decade [1,2]. Bone material for graft come either from femoral heads removed during hip arthroplasty surgery or large bone segments such as parts of long bones or hemipelves from cadaver donors. Despite an increasing viral safety level caused by donor screening, serological or polymerase chain reaction testing, a small but irreducible risk of disease transmission and particularly of viral origin is always present. HIV-1 transmission by bone allografts was published twice whilst three cases of transmission of the hepatitis C virus by

* Corresponding author. Tel: +33 5 63 49 3141;
fax: +33 5 63 49 3099; e-mail: jfages@enstimac.fr

tissue transplantation were reported in the literature [3].

Additionally, it was reported that the integration of massive bone grafts within newly formed bone is partial if any. This poor integration was attributed to the immunological mismatching between the donor and the receiver [4,5]. Antigenic matching between donor and host is not checked for bone grafting. The surface of bone marrow cells are rich in HLA (Human Leucocyte Antigen) determinants. They are responsible for the recognition of these cells by the host immunitary system as belonging to another person. The numerous remnants of bone marrow cell membranes in the donor bone [6] are responsible for the immune response against it and the rejection reaction of the graft [7,8]. It was reported that bone marrow removal may enhance osseointegration of bone allografts [9]. It is noteworthy that most bone allografts do not follow such a process.

There is a need to increase both viral safety and graft integration to reduce the risk of graft fracture, infection and osteolysis. A new bone tissue process was described for the first time by Fages et al. in 1994 [10,11]. Its main characteristics lie in the use of supercritical fluid technology.

Because of their unique properties of diffusivity, density and viscosity, supercritical fluids are used as selective extraction solvents [12]. They are particularly adapted for extraction of components entrapped in a solid microporous structure which is the case of bone tissue. Carbon dioxide is a good solvent of non-polar molecules, it is non-toxic, returns to gaseous state at atmospheric pressure and its critical pressure and temperature ($P_c = 7.38$ MPa; $T_c = 31.1°C$) are relatively easy to reach [13]. It is therefore this compound which was chosen for bone tissue extraction.

Supercritical CO_2 extraction leads to a total range in-depth delipidation of the bone porosity. Three additional stages, facilitated by the delipidation, complete the bone processing: hydrogen peroxide is used for protein and cellular debris elimination. Sodium hydroxide for the inactivation of potentially present prions [14], and ethanol for final cleansing and for its well-known virucidal properties [15].

2. Materials and Methods

2.1. Bone processing steps

Step 1. Supercritical extraction: A pilot plant (Separex, Champigneulles, France) for batch extraction and separation was used. In this apparatus, cooled liquid carbon dioxide is pressurised by a metallic membrane pump and heated to the extraction temperature. The extraction vessel with a volume of 2.5 l is loaded with a titanium basket containing the bone samples to be treated. The extraction vessel is followed by a series of three thermostatically controlled separation vessels, in which pressure is adjusted by needle valves. At the outflow of the last separator, CO_2 is liquefied and recycled. Except for the loading and unloading operations, all the operations were remote controlled.

Operating conditions were: CO_2 flow rate 2 kg h^{-1}; pressure 250 bar; extraction temperature 50°C. Time: 10 min per gram of bone.

Step 2. Hydrogen peroxide processing: concentration 35% (w/w) at 40°C for 2 h.

Step 3. Sodium hydroxide processing: concentration 1 M at 20°C for 1 h. Samples were then neutralised in NaH_2PO_4 (12 g l^{-1}).

Step 4. Ethanol processing: concentration 95% at 20°C for 3 h followed by: concentration 100% at 20°C for 2 h.

2.1.1. Viral safety

Viral inactivation of bone allografts is generally attained by using one of the following processes: β or γ irradiation, ethylene oxide or autoclaving. None of these methods have proved to be totally satisfactory, either caused by the resistance of some viruses to physico-chemical processing or caused by the loss of inductive or mechanical properties of the graft material [16–19].

One of the principal approaches to control potential viral contamination of biologicals is to test the capacity of the preparation process to remove or inactivate viruses. Each of these four previously-mentioned processing stages were checked for virus inactivation and/or removal by spiking of a significant amount of relevant virus in a pre-processed bone sample, performing the

step to be evaluated and recovering the virus from the post-processed bone sample. The viral inactivation and/or elimination of each stage may be quantified by calculating a reduction factor which is the $\log_{10}$ of the ratio initial virus load/final virus load.

Four viruses chosen according to the European community guideline on virus inactivation validation [20] were used: HIV-1 (RNA enveloped retrovirus), Sindbis virus (RNA enveloped virus, model for Hepatitis C virus), Polio Sabin virus (RNA non-enveloped virus, model for Hepatitis A virus) and Pseudorabies virus (DNA enveloped virus as the Hepatitis B virus).

The ability of each step to inactivate and/or eliminate the viruses was evaluated from two pieces of human femoral head treated independently. The spiked and control samples obtained from the bone pieces, were assayed for viral infectivity by plaque assays for Sindbis, Polio and PRV [21] and by infectivity assay for HIV-1 [22]. For each step and for each virus, a detection limit (DL) of the titration assay which depends on the cytoxicity of the tested solution added to the cells was calculated. When no infectious unit was recovered from a spiked and processed bone, the result was therefore quantified as lower than DL and the corresponding reduction factor as higher than the calculated value.

2.1.2. *In vivo implantation*

Such treated allografts were implanted in sheep femoral condyles and tibial epiphyses and compared to non-treated allografts. Surgical procedure: Adult sheep were used for the experimentation and were housed in the facilities of the surgical department of the National Veterinary School of Toulouse (France) according to institutional guidances for animal welfare. After the sheep was anaesthetised, a lateral approach was made and a 6 mm hole was drilled either into the femoral condyle or into the proximal epiphysis. The implant was aseptically introduced and the absence of micro-movement was checked. Two animals were implanted with each implant for each implantation period. After implantation periods of 1, 4 and 8 months, animals were sacrificed using a pentobarbital injection and the femoral condyles and tibial epiphyses removed.

2.1.3. *Histological analysis*

The explants were fixed in a 4% buffered formaldehyde solution for 5 days and were dehydrated in increasing alcohol concentrations. They were embedded in polymethylmethacrylate (PMMA) and 2 mm thick sections were made using a low speed cooled diamond saw. They were set in the surface of a polystyrene block, then, they were ground on silicon carbide discs until a 50 μm thickness. They were then stained with a toluidine blue solution after having etched in a 2% formic acid solution followed by a 20% methanol solution. They were observed with a Reicher Polyvar microscope.

3. Results and discussion

3.1. *Viral inactivation*

The four stages of the bone treatment process were evaluated independently, with bone samples treated up to the step to be assayed i.e. treated by stages 1 and 2 for an evaluation of stage 3. The assumed elimination or inactivation parameters of each step were clearly independent and the step-specific reduction factors can be added in order to calculate a cumulative reduction factor Rc which is characteristic of the complete process for each virus. As shown in Table 1, the global inactivation is extremely high with reduction factors ranging from over 14 to over 24. The reason for such a broad range lies in the various abilities of each virus to persist in the bone pieces in the positive controls.

The four steps of the bone manufacturing process efficiently eliminate or inactivate the tested viruses since reduction factors higher than 4 logs were generally obtained. For HIV-1 and stages 2 and 3, such a high value could not be demonstrated since a poor detection limit led to reduction factors of >3.6 and >2.2, respectively. These low limits were not process-dependent but linked with technical limitations of the assays.

The Polio Sabin virus which is non-enveloped

Table 1
Reduction factors associated with four stages of the bone process. Reduction factors are the $\log_{10}$ of the ratio of the virus load before and after the stage to be evaluated

	HIV-1	Sindbis	Polio Sabin	Pseudo rabies
SC CO_2	>4.05	>4.31	>6.58	>4.02
H_2O_2	>3.61	>4.89	>7.05	>4.71
NaOH	>2.19	4.08	4.25	>4.66
C_2H_5OH	>4.37	4.98	>6.6	>4.21
cumulative Rc	>14.22	>18.26	>24.48	>17.60

and more resistant to physico-chemical treatments demonstrated the relative efficiency of the four stages. It is noteworthy that the sodium hydroxide step is the least efficient technique for viral inactivation. This step however was introduced in bone allograft processing to prevent the risk of transmission of spongiform encephalopathies such as Creutzfeld–Jakob Disease. The three other steps led to a total inactivation of the Polio virus, giving very high reduction factors in the range 6 to 7 $\log_{10}$.

Despite the presence of a stage of prion inactivation, there is no direct proof that this process is efficient in inactivating prions in bone tissue, although bone belongs to a class of tissue for which no infectivity has ever been demonstrated in animal models [23]. Prion transmission was highlighted in Europe following the possible transmission of the bovine spongiform encephalopathy to humans revealed by the British Ministry of Health and through the iatrogenic transmission of Creutzfeld–Jakob Disease from contaminated growth hormone extracted from cadaver pituitary glands [24].

3.2. Comparison with gamma irradiation and ethylene oxide methods

Very few data on viral inactivation in bone tissue are available. Fideler et al. [25] showed that 25 kGray, the recommended dose for medical products, is not sufficient to eliminate the DNA of HIV-1 in ligament-bone grafts obtained from infected cadavera. They recommend a minimum dose of 30 kGy. It is difficult, however to compare with our data because the level of infection in the bone was not measured in this study. In another study, Campbell et al. [26] showed that a dose of at least 25 to 30 kGy was necessary to sterilize a bone piece spiked with 700 ml of a 5×10^4 virus ml^{-1} culture. However, they stressed the fact that high doses may be deleterious to the structural integrity of allograft bone. Both studies confirmed that viruses are radio-resistant and that classical doses used in bone bank procedures may not be sufficient.

The use of ethylene oxide was even less documented. Mellonig et al. [27] cited unpublished data in which ethylene oxide inactivated HIV within dense cortical bone. However, no figure was given.

3.3. Graft functionality

An obvious difference in the integration of the treated material compared to the non-treated control material appeared as early as one month after implantation. After an eighteen-week implantation time, the external trabeculae surface was coated by a newly formed bone layer that was composed of immature bone with one or several layers of active osteoblasts as shown in Fig. 1. The material pores were invaded by a highly vascularized stromal tissue containing some plasmocytes and lymphocytes.

Non-treated implants were separated from the recipient bone by a thick layer of connective tissue which entered in the pores of the implant. This connective tissue contained many plasmocytes and lymphocytes. Most of the pores were invaded by this immune cell infiltrate indicating a strong immunological reaction against the implant as shown in Fig. 2. It is noteworthy that the internal pores that were not filled with this infiltrate were filled with remnants of necrotic donor tissue.

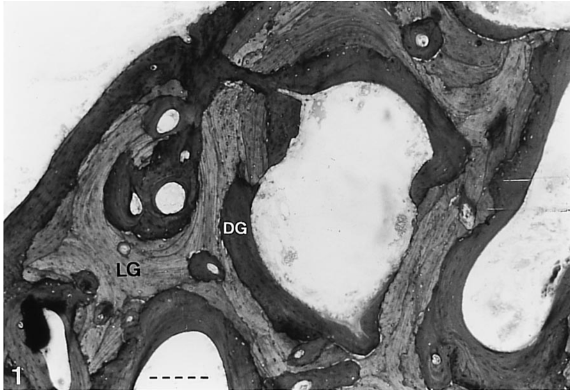

Fig. 1. Histological section of a treated bone allograft (LG) after an eighteen-week implantation time. The implanted bone is totally layered by a newly formed bone (DG) and fragmented by the resorption process of the implant which is being replaced by the host bone. Some fragments are phagocytosed by macrophages located in bone marrow cavities (white). This process of bone integration is known as creeping substitution. No inflammatory reaction suggesting an immunitary reaction against the implant was noted. Bar: 200 μm.

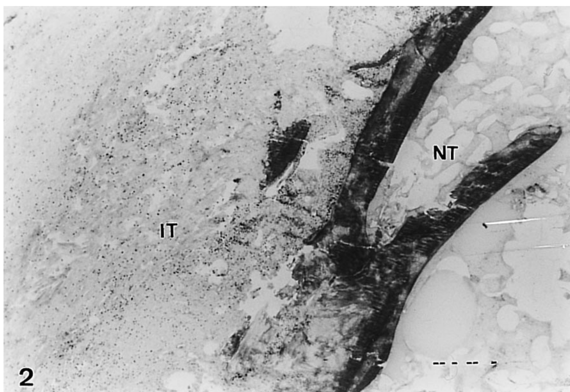

Fig. 2. Histological section of a non-treated bone allograft (dark) after a four-week implantation time. The implant is fragmented by the resorption process and separated from the recepient bone by a connective tissue (IT) constituted of plasmocytes and lymphocytes (black dots) suggesting a strong immunological reaction to the implant. Pores are filled by necrotic tissue remnants (NT). Bar: 400 μm.

At four months of implantation, treated material was totally integrated within newly formed bone. Most of the bone surface was coated with a layer of recipient bone. The pores were filled with a stromal tissue containing a few plasmocytes islets. Some bone fragments having a size of a few microns were found phagocytosed in histiocytes disseminated in the connective tissue. Remodelling signs of the newly formed bone were shown, resorption marks were found in the implanted bone with newly formed bone ingrowth characterising the process of creeping substitution. All the non-treated allografts were resorbed to a great degree and were replaced by a loose connective tissue with some remnants of bone matrix. Some islets of plasmocytes were found in this tissue grouped around small vessels.

Eight months after implantation, treated materials could not be distinguished from the bone in which they had been implanted. The remodelling process having taken place at the implant contact led to its resorption following its integration, and then to its replacement by newly formed bone. In contrast, all but one of the non-treated allografts were almost completely resorbed and replaced by a loose connective tissue leaving a cavity in the implanted bone.

Lipid extraction of bank bones has already been proven to increase the rate of incorporation [28]. This experiment gave clear confirmation of such an effect, because the allograft treatment led to an enhanced integration potential of the graft. A lowering of the immunological reaction of the host against the graft was shown histologically. It is known that activation of the immune cells involved in the immune reaction can trigger an activation of the osteoclasts [29]. The osteolysis of the non-treated allografts is, thus, consistent with the presence of immune cell infiltrate within the material pores. The only difference between the two implant types tested lies in the presence or absence of the bone marrow cavity components, the components of the bone extracellular matrix, particularly the type I collagen matrix, being unaltered by the treatment. Therefore, the immunological reaction against the bone allograft can be attributed to the bone marrow components and particularly the antigenic molecules of the bone marrow cell membranes.

4. Conclusions

This study indicates that this process (i) increases the viral safety of bone grafts and (ii)

decreases the immunogenicity of such grafts thus helping the functionality of such materials. Therefore, this process could become a new way for processing bank bones alternatively or additionally to the existing procedures.

It is a subject of interest to be able to improve the procedures of bone banking at a time when, because of new regulations and laws ratified by several European governments, a shortage in human bone materials is to be expected.

References

[1] A.D. Saies, D.C. Davidson, Femoral head allograft bone banking, Aust. N. Z. J. Surg. 60 (1990) 267.

[2] T.I. Malinin, O.V. Martinez, M.D. Brown, Banking of massive osteoarticular and intercalary bone allografts. 12 year's experience, Clin. Orthoped. Rel. Res. 197 (1984) 44.

[3] W.W. Tomford, Transmission of disease through transplantation of musculosketelon allografts, J. Bone Joint Surg. 77A (1995) 1742.

[4] M.C. Horowitz, G.E. Friedlander, Immunologic aspects of bone transplantation. A rationale for future studies, Orthop. Clin. North. Am. 18 (1987) 227.

[5] G.E. Friedlander, Bone allografts: the biological consequence of immunological events, J. Bone Joint Surg. 73A (1991) 1119.

[6] S. Stevenson, M.C. Horowitz, The response to bone allograft, J. Bone Joint Surg. 74A (1992) 939.

[7] H. Skojdt, D.E. Hughes, P.R.M. Dobson, R.G.G. Russell, Constitutive and inducible expression of HLA class II determinants by human osteoblast-like cells *in vitro*, J. Clin. Invest. 85 (1990) 1421.

[8] G.J. Hämmeling, Tissue distribution of Ia antigens and their expression on lymphocyte subpopulations, Transplant. Rev. 30 (1976) 64.

[9] P. Aspenberg, K. Thoren, Lipid extraction enhances bank bone incorporation. An experiment in rabbits, Acta Orthop. Scand. 61 (1990) 546.

[10] J. Fages, A. Marty, C. Delga, J.S. Condoret, D. Combes, P. Frayssinet, The use of supercritical CO_2 for bone delipidation, Biomaterials 15 (1994) 650.

[11] J. Fages, A. Marty, J.S. Condoret, Processing of bone substitute with supercritical carbon dioxide, in: M. Perrut, G. Brunner (Eds.) Proceedings of the third International Symposium on Supercritical Fluids, vol 2, 1994, p.253.

[12] J.M. Wong, K.P. Johnston, Solubilisation of biomolecules in carbon dioxide based supercritical fluids, Biotech. Prog. 2 (1986) 29.

[13] A.J. Hyatt, Liquid and supercritical carbon dioxide as organic solvents, J. Org. Chem. 49 (1984) 5097.

[14] D.M. Taylor, H. Fraser, I. Mc Connell, D.A. Brown, K.L. Brown, K.A. Lamza, G.R.A. Smith, Decontamination studies with the agents of bovine spongiform encephalopathy and scrapie, Arch. Virol. 139 (1994) 313.

[15] J.T. Mellonig, A.B. Prewett, M.P. Moyer, HIV inactivation in a bone allograft, J. Periodontol. 63 (1992) 979.

[16] M.J. Doherty, R.A.B. Mollan, D.J. Wilson, Effect of ethylene oxide sterilisation on human demineralized bone, Biomaterials 14 (1993) 994.

[17] D.G. Campbell, P. Li, A.J. Stephenson, R.D. Oakeshott, Sterilisation of HIV by gamma irradiation: a bone allograft model, Int. Orthop. 18 (1994) 172.

[18] H. Sugiura, K. Sato, Y. Rong, S. Yamamura, K. Nakanishi, T. Ito, A mechanical and biological study of surface demineralized and heat-treated cortical bone, Eur. J. Exp. Musculoskel. Res. 3 (1994) 157.

[19] K.J. Hallfeldt, H. Stützle, M. Puhlmann, S. Kessler, L. Schweiberer, Sterilization of partially demineralized bone matrix: the effects of different sterilization techniques on osteogenic properties, J. Surg. Res. 59 (1995) 614.

[20] EC regulatory document. Validation of virus removal and inactivation procedures. Note for guidance, III/8115/89-EN. Biologicals, 20 (1992) 159.

[21] S. Harada, Y. Koyanagi, N. Yamamoto, Infection of HTLV-III/LAV in HTLV-I carrying cells MT-2 and MT-4 and application in a plaque assay, Science 229 (1985) 563.

[22] H. Gregersen, L. Wege, K. Preiss, J. Jentsch, Detection of human immunodeficiency virus and other retroviruses in cell culture supernates by a reverse transcriptase microassay, Virol. Meth. 19 (1988) 161.

[23] J. Paul, La manipulation du prion au laboratoire, Path. Biol. 43 (1995) 121.

[24] T. Billette de Villemeur, P. Beauvais, M. Gourmelon, J.M. Richardet, Creutzfeldt–Jakob disease in children treated with growth hormone, Lancet 337 (1991) 864.

[25] B.M. Fideler, C.T. Vangsness, T. Moore, Z. Li, S. Rasheed, Effects of gamma irradiation on the human immunodeficiency virus. A study in frozen human bone-patellar ligament-bone grafts obtained from infected cadavera, J. Bone Joint Surg. 76A (1994) 1032.

[26] D.G. Campbell, P. Li, A.J. Stephenson, R.D. Oakeshott, Sterilization of HIV by gamma irradiation, Int. Orthoped. 18 (1994) 172.

[27] J.T. Mellonig, A.B. Prewett, M.P. Moyer, HIV inactivation in a bone allograft, J. Periodontol. 63 (1992) 979.

[28] K. Thoren, P. Aspenberg, K.G. Thorngren, Lipid extracted bank bone, Clin. Orthoped. Rel. Res. 311 (1995) 232.

[29] T.J. Chambers, The cellular basis of bone resorption, Clin. Orthoped. Rel. Res. 151 (1980) 283.