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## Supercritical Processing Enhances Viral Safety and Functionality of Bone Allografts

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A new bone tissue process using supercritical carbon dioxide extraction has been checked for viral inactivation and allografts, thus processed, for bone integration. Four viruses, human immunodeficiency virus type I (HIV-1), Sindbis virus, Polio Sabin type I virus and Pseudorabies virus (PRV) and four processing stages, supercritical CO<sub>2</sub>, hydrogen peroxide, sodium hydroxide and ethanol treatments were tested. The cumulated reduction factors (log<sub>10</sub>) were the following: >18.2 for Sindbis virus, >24.4 for Poliovirus, >17.6 for PRV and >14.2 for HIV-1. Such processed allografts were implanted into sheep leading to a much faster osseointegration in comparison with non-treated allografts. Better graft incorporation in addition to viral safety suggest that this process could become a new way for processing bank bones, alternatively or additionally, to the procedures presently used.

### Introduction

Due to 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 have been tremendously increased over the past decade [1,2]. Bone material for graft come either from femoral heads removed during hip arthroplasty surgery or from cadaver donors for large bone pieces such as part of long bones or hemipelves. Despite an increasing viral safety level due to donor screening, serological or PCR testing a small but irreducible risk of disease transmission and particularly of viral origin is always present. HIV-1 transmission by bone allografts has been published twice whilst three cases of transmission of the hepatitis C virus by tissue transplantation have been reported in the literature [3].

Additionally, it has been reported that the integration of massive bone grafts within newly formed bone is partial if any. This poor integration has been attributed to the immunological mismatching between the donor and the receiver [4,5]. Although HLA determinants of the graft are not checked before implantation, this thesis was asserted by the presence of remnants of donor bone marrow cells within the graft bone marrow cavities before implantation [6], these cells being rich in class I and II membrane glycoproteins of the HLA system [7,8]. It has been 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. Its main characteristics lies in the use of supercritical fluid technology [10].

Due to their unique properties of diffusivity, density and viscosity, supercritical fluids are used as selective extraction solvents [11]. 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<sub>c</sub> = 7.38 MPa ; T<sub>c</sub> = 31.1°C) are relatively easy to reach [12]. It is therefore this compound which was chosen for bone tissue extraction.

Supercritical CO<sub>2</sub> 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 [13], and ethanol for final cleansing and for its well-known virucidal properties [14].

### Materials and Methods

#### Bone processing steps

Step 1. Supercritical extraction : A pilot plant (Separex, Champigneulle, 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<sub>2</sub> is liquefied and recycled. Except for the loading and unloading operations, all the operations were remote controlled.

Operating conditions were: CO<sub>2</sub> flow rate 2kg/h; pressure 250 bars; extraction temperature 50°C. Time: 10 min per gram of bone.

Step2. Hydrogen peroxide processing : concentration 35% (w/w) at 40°C for 2 h.

Step3. Sodium hydroxide processing : concentration 1M at 20°C for 1 h. Samples were then neutralised in NaH<sub>2</sub>PO<sub>4</sub> (12g/l)

Step4. Ethanol processing : concentration 95% at 20°C for 3 h followed by : concentration 100% at 20°C for 2 h.

Viral safety. Viral inactivation of bone allografts is generally attained by using one of the following processes:  $\beta$  or  $\gamma$  irradiation, ethylene oxide or autoclaving. None of these methods have proved to be totally satisfactory, either due to the resistance of some viruses to physico-chemical processing or due to the loss of inductive or mechanical properties of the graft material [15-18].

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 above-mentioned processing stages have been 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<sub>10</sub> of the ratio initial virus load/final virus load.

Four viruses chosen according to the EC guideline on virus inactivation validation [19] 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 samples obtained from the bone pieces, spiked and then treated or not were assayed for viral infectivity by plaque assays for Sindbis, Polio and PRV [20] and by infectivity assay for HIV-1 [21]. For each step and for each virus, a detection limit (DL) of the titration assay depending on the cytotoxicity 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.

In vivo implantation. Such treated allografts were implanted in sheep femur condyles and tibial epiphyses and compared to non treated allografts. Surgical procedure: Adult sheep were used for the experimentation and were stabulated in the facilities

of the surgical department of the National Veterinary School of Toulouse (France) following institutional guidances for animal welfare. After sheep was anaesthetised, a lateral approach was made and a 6 mm hole was drilled either into femoral condyle or into 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.

Histological analysis. The explants were fixed into a 4% buffered formaldehyde solution for 5 days and were dehydrated into increasing alcohol concentrations. They were embedded into polymethylmethacrylate (PMMA) and 2 mm thick sections were made using a low speed cooled diamond saw. They were settled at the surface of a polystyrene block, then, they were ground on silicon carbide discs until a 50  $\mu$ m thickness. Then, they were 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.

## Results and discussion

Viral inactivation. The four stages of the bone process were evaluated independently, with bone samples treated till the step to be assayed. The putative 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.

	HIV-1	Sindbis	Polio Sabin	Pseudo rabies
SC CO <sub>2</sub>	>4.05	>4.31	>6.58	>4.02
H <sub>2</sub> O <sub>2</sub>	>3.61	>4.89	>7.05	>4.71
NaOH	>2.19	4.08	4.25	>4.66
C <sub>2</sub> H <sub>5</sub> OH	>4.37	4.98	>6.6	>4.21
cumulative Rc	>14.22	>18.26	>24.48	>17.60

Table 1. Reduction factors associated with four stages of the bone process. Reduction factors are the log<sub>10</sub> of the ratio of the virus load before and after the stage to be evaluated.

The four steps of the bone manufacturing process are efficient to 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 and more resistant to physico-chemical treatments allowed to distinguish the relative efficiency of the four stages. It is noteworthy with this virus that the sodium hydroxide step is the less efficient 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 [22]. Prion transmission came under the lights in Europe following the possible transmission of the BSE 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 [23].

**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. The external trabeculae surface was coated by a newly formed bone layer constituted of immature bone edged by one or several layers of active osteoblasts (Fig.1).



Figure 1. Histological section of a treated bone allograft (light grey) after an eighteen-week implantation time. The implanted bone is totally layered by a newly formed bone (dark grey) 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.

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 cells infiltrate indicating a strong immunological reaction against the implant (Fig. 2). It was interesting to notice that the internal pores that were not filled with this infiltrate were filled with remnants of necrotic donor tissue.



Figure 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 recipient bone by a connective tissue (IT) constituted of plasmocytes and lymphocytes (black dots) suggesting a strong immunitary reaction against the implant. Pores are filled by necrotic tissue remnants (NT).

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 ingrowths characterising the process of creeping substitution. All the non treated allografts were mostly totally resorbed and 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. On the contrary, all but one 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 proven to fasten incorporation [24]. This experiment gave clear confirmation of such an effect, this allograft treatment leading to 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 immunitary reaction trigger an activation of the osteoclasts [25]. 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 ECM, 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 HLA complex of the bone marrow cell membranes.

### 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, due to new regulations and laws emitted by several European governments, a shortage in human bone materials is to be expected.

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