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(RESEARCH ARTICLE)

Evaluation of the biomechanical and structural properties of bone allografts treated with a new cleaning process

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Abstract

The use of allograft bone is becoming increasingly common. The intrinsic mechanical and structural properties of the graft are of major importance for osseointegration. Current cleaning treatments using chemical or physical products increase biosafety but may disturb these bone characteristics. A new cleaning treatment for cancellous and cortical bone by simple mechanical washing (sonication and centrifugation) and supercritical carbon dioxide (scCO2) treatment was developed. The mechanical and structural properties of allografts cleaned with this treatment were compared with those obtained after preservation by freezing at −80°C. Three-point bending, compression and hardness tests were performed for biomechanical analysis. The structural characteristics of the allografts were evaluated by microscanner (histomorphometry) and scanning electron microscopy (surface analysis). All the data showed that the cleaned bone was generally stiffer owing to delipidation but its structure remained similar to that of the frozen bone. Bone cleaned by this new treatment thus displayed mechanical and structural properties close to those of frozen bone.

Keywords: Bone allograft; Cleaning process; Supercritical carbon dioxide; Mechanical analysis.

1. Introduction

Bone tissue is a complex material owing to its composite (mineral and organic phases) and multi-scale structure, which give its elasto-plastic properties and anisotropic behavior, together with biological properties. When bone substance is lost, a bone allograft can be used to restore it. Such a graft plays both mechanical and osteoconductive roles. The mechanical and biological properties of a graft are closely linked. In the medium-to-long term, only its successful osseointegration will guarantee mechanical performance.

The intrinsic structural properties of a graft are of major importance during the first few months after grafting. On a macroscopic scale, the graft (in association with the osteosynthesis or prosthesis) will have to withstand cyclic local stresses. There is risk of graft fracture by fatigue in the short-to-medium term. At the micro- and nanoscopic scales, the microarchitectural characteristics of the graft will affect osseointegration and biomechanical capacities. It is generally considered that larger pore size and pore density promote bone regrowth but to the detriment of mechanical properties [1,2]. The specific surface area of the graft is also probably of prime importance, as bone regrowth is said to occur by epitaxy [3].

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Various current additional treatments result in structural and biomechanical modifications of cancellous or cortical grafts, altering both their organic and inorganic scaffolds. For example, gamma irradiation (at the usual dose of 25 kGy) reduces the flexural strength of cortical bone by about 20% [4,5]. Mechanical cleaning and freeze-drying cause an increase in Young's modulus (stiffening) of cancellous bone [6]. Chemicals (solvents, alcohol, detergents, urea, sodium hydroxide, hydrogen peroxide, etc.) alter the protein structure and fibrillar collagen, which can lead to a 35% loss of mechanical capacities in some procedures [7].

In this context, we developed a new chemical-free complementary treatment process for both cancellous and cortical bone. It is based on the association of non-aggressive mechanical cleaning (ultrasound baths in saline and centrifugations) and extraction using supercritical carbon dioxide (scCO2). The whole treatment is carried out at a temperature lower than or equal to 40° in order not to denature the collagenous framework.

The effects on the mechanical and structural properties of the bone graft of this new chemical-free treatment had not hitherto been evaluated.

The main objective of this study was to evaluate the mechanical and structural characteristics of bone treated with this new process ("scCO2 bone"), versus those of bone preserved by freezing ("F bone").

The hypothesis was that there would be no difference between these two bone groups.

2. Material and methods

2.1. Ethical authorization

All the tissues and cells of human origin came from the Osteobank tissue bank and the Orthopedics and Traumatology Department of the University Hospital, Clermont-Ferrand. Authorization for research use was granted (DC-2021-4555).

2.2. Study of biomechanical properties by macroscopic mechanical tests

Mechanical three-point bending and compression tests were performed on an MTS 20M hydraulic tension-compression/torsion device, equipped with a three-point bending platform (range 40 mm) and compression plates (Fig. 1). The unit was controlled by management software throughout the testing and results processing procedure. A 500 daN load cell was available for these destructive tests. Strain measurement was tracked from the crosshead recording (resolution 100 µm). The crosshead displacement speed was set during the tests at 3 mm/min. To facilitate analysis of the results and allow comparison of mechanical responses between different specimens, the results were recorded as conventional stresses and strains, calculated from the apparent specimen size.

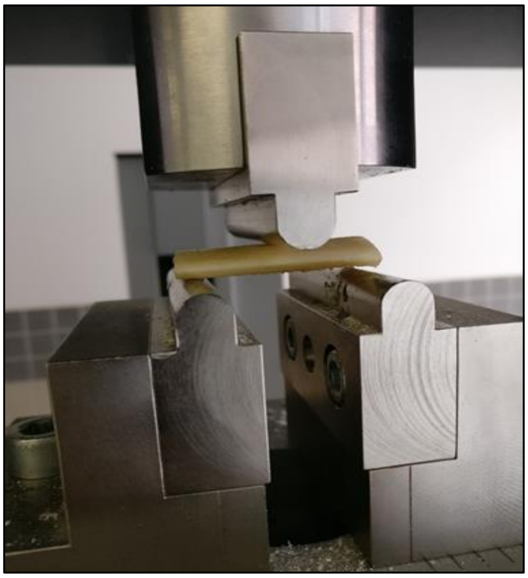

Figure 1 Three-point bending test (MTS 20M traction/compression device) on an scCO2-treated cortical bone rod.

The three-point bending test gave a stress-strain curve, Young's modulus of elasticity (MPa), the associated stress at the point of failure (MPa), and the strain at failure (%).

For the compression test, apparent modulus (MPa), maximum apparent stress (MPa) and maximum apparent deformation (%) were measured.

These tests were performed on 10 cancellous bone samples and 10 cortical femur bone samples from 10 different donors for each of the F bone and scCO2 bone groups. For the bending tests, the specimens were slabs measuring 60 × 10 × 10 mm, and for the compression tests they were cubes measuring 10 ×10 ×10 mm.

Samples from the F bone group were thawed according to a protocol similar to that used in clinical practice, namely progressively, by thawing at −20°C for 6 h and then at room temperature in saline for 20 minutes.

A third mechanical surface hardness test was performed. Shore A hardness was measured

on a Shore hardness tester equipped with a specific head. This test was performed on 75 samples (slabs measuring 20 ×10 ×10 mm) for each bone group.

2.3. Study of histomorphometric properties by microscanner

Analyses were performed with an XCT-3000 pQCT micro-CT (Stratec Biomedical) using ImageJ software (National Institutes of Health) and the BoneJ plugin. The maximum resolution of the micro-CT was 137 µm. The X-ray power was 12.5 W, the tube voltage was 61.2 kV, and the current was 0.204 mA.

Five samples (A, B, C, D, E) of cancellous bone measuring 10 × 10 × 30 mm were collected from the lower end of the femur of five different donors (three males and two females with mean age 56 ± 12 years). Each sample was then cut in half (dimensions 10 × 10 × 15 mm). One fragment was stored by freezing at −80°C (F bone group). The other was cleaned by scCO2 treatment and then stored at room temperature (scCO2 bone group). Measurements were made on five successive sections 2 mm apart for each fragment.

The following histomorphometric analyses were performed:

- Morphological analyses:
    - trabecular bone mineral density (BMD) (in mg/cm^3),
    - BV/TV ratio, corresponding to the volume of mineralized bone (BV) divided by the total volume (TV) (in %),
    - thickness of the bone trabeculae (Tb.th) (in mm),
    - space between the bone trabeculae (Tb.sp) (in mm).
- Topological analysis:
    - anisotropy (the preferential directional organization of a material in space: an anisotropy equal to 0 means that there is no specific orientation of the trabeculae, an anisotropy equal to 1 it means that there is a specific orientation of all the trabeculae),
    - connectivity, representing the number of connections between the bone trabeculae.
- Texture analysis:
    - the fractal dimension, reflecting the three-dimensional micro-architectural complexity of a surface,
    - the structure model index (SMI), which quantifies the morphology of the structure according to whether it is made preferentially of plates or rods (a plate structure will have a value close to 0 and a rod structure a value close to 3).

2.4. Surface study by scanning electron microscopy

Each sample was fixed, after washing with PBS, with 1.6% glutaraldehyde in 0.2 M sodium cacodylate buffer pH 7.4 (Delta Microscopies) at 4°C. Images were recorded by the Centre Imagerie Cellulaire Santé (CICS) of the Faculty of Medicine, Clermont-Ferrand, with an XL 30 SEM (Philips).

The purpose of SEM analysis was to examine the microarchitecture and surface of cancellous bone grafts from the F bone and scCO2 bone groups at three different scales (×30, ×100, and ×1000 magnification). After subjective visual assessment of surface appearance, manual counting of the number of large and small medullary cavities (greater than or less than 25 µm, respectively) was performed twice (one week apart) for each bone group on five randomly selected ×100 magnification images.

2.5. Statistics

Statistical analyses were performed with Excel software (Microsoft). The results were expressed as mean and standard deviation (SD) and compared using Student's *t* test. The significance level chosen was $p < 0.05$.

For the histomorphometric analyses by microscanner, the results of each variable are presented as an overall average and SD of the five sections of all samples.

When the numbers of cavities per SEM image were counted, a correlation coefficient between the two-count series was calculated.

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3. Results

3.1. Study of biomechanical properties by macroscopic mechanical tests

Overall, both cancellous bone (Table 1) and cortical bone (Table 2) treated with scCO2 had a higher modulus of elasticity and stress at failure, but lower deformation than frozen bone.

Table 1 Results of mechanical tests on cancellous bone.

Mechanical characterization	**F bone**	**scCO2 bone**	***p***
Three-point bending			
Young's modulus (MPa)	197.7 ± 103.3	261.3 ± 197.9	0.09
Stress at failure (MPa)	6.7 ± 4.4	9.7 ± 4.8	0.11
Deformation at failure (%)	9.2 ± 7.1	7.9 ± 6.2	0.57
Compression			
Apparent modulus (MPa)	114.7 ± 69.0	354.9 ± 261.6	0.004
Max. stress (app) (MPa)	6.1 ± 3.5	11.2 ± 6.8	0.01
Max. deformation (app) (%)	8.5 ± 3.4	6.2 ± 3.8	0.11
Shore A hardness	70.5 ± 8.8	82.6 ± 9.0	0.01

Table 2 Results of mechanical tests on cortical bone.

Mechanical characterization	**F bone**	**scCO2 bone**	***p***
Three-point bending			
Young's modulus (MPa)	393.3 ± 362.8	891.0 ± 1174.5	0.04
Stress at failure (MPa)	37.6 ± 20.5	45.9 ± 26.6	0.02
Deformation at failure (%)	23.5 ± 13.7	10.9 ± 5.8	0.001
Compression			
Apparent modulus (MPa)	1517.8 ± 809.3	1510.3 ± 414.4	0.6
Max. stress (app) (MPa)	NA	NA	
Maw deformation (app) (%)	NA	NA	
Shore A hardness	86.3 ± 5.4	84.8 ± 8.8	0.08

These differences were significant only for cancellous bone in compression tests, and only for cortical bone in flexion tests.

Hardness was also significantly higher for cancellous treated bone (approx. +15%, *p* = 0.01), but not for cortical bone.

In other words, the treated bone was stiffer, stronger, and harder but less deformable (and so more brittle) than the frozen bone.

3.2. Study of structural properties by microscanner

The results of the average of the five samples (average of A+B+C+D+E) are presented in Table 3. No significant difference was found between the two groups of bones in morphological, topological, or textural variables.

Analysis of the results of each bone sample yielded further information:

No significant difference was found over the five samples for the variables trabecular density and BV/TV ratio.

The thickness of the trabeculae (Tb.th) was significantly different for three samples. Trabeculae were thicker (approx.+25%) in the scCO2 bone group for two samples (*p* = 0.05) but were thinner (approx. −30%) for one other sample (*p* = 0.04).

The inter-tabular space (Tb.sp) was significantly greater (approx. +30%) in the F bone group (*p* = 0.02) for one sample.

Connectivity was very significantly lower (about −55%) in the scCO2 bone group (*p* = 0.001) for one sample.

The fractal dimension was significantly higher (approx. +5%) in the scCO2 bone group for two samples (*p* = 0.03 and 0.05 respectively).

The structure model index was significantly different for three samples. It was lower (approx. −20%) in the scCO2 bone group for two samples (*p* = 0.04 and 0.03 respectively), and higher (about +120%) for one other sample (*p* = 0.001).

Table 3 Average values of the different histomorphometric variables of all the cancellous bone samples.

	F bone	**scCO2 bone**	***p***
Trabecular density (mg/cm^3)	143.16 ± 26.79	134.92 ± 22.76	0.25
BV/TV (%)	41.25 ± 5.00	44.26 ± 8.00	0.15
Tb.th (mm)	0.493 ± 0.16	0.502 ± 0.08	0.81
Tb.sp (mm)	0.795 ± 0.35	0.795 ± 0.34	0.99
Anisotropy	0.681 ± 0.27	0.653 ± 0.31	0.74
Connectivity	674.205 ± 410.69	474.245 ± 338.74	0.07
Fractal dimension	2.898 ± 0.32	2.913 ± 0.28	0.87
Structure model index	3.147 ± 0.79	3.347 ± 0.52	0.29

3.3. Surface study by scanning electron microscopy

At low and medium magnifications (Fig. 2 and 3), the images of the F bone and scCO2 bone groups were closely similar. The trabecular structure was unchanged. The surface of the trabeculae in the scCO2 bone group appeared rougher (Fig. 2c) and the intertrabecular cavities emptier than in the F bone group (Fig. 3d).

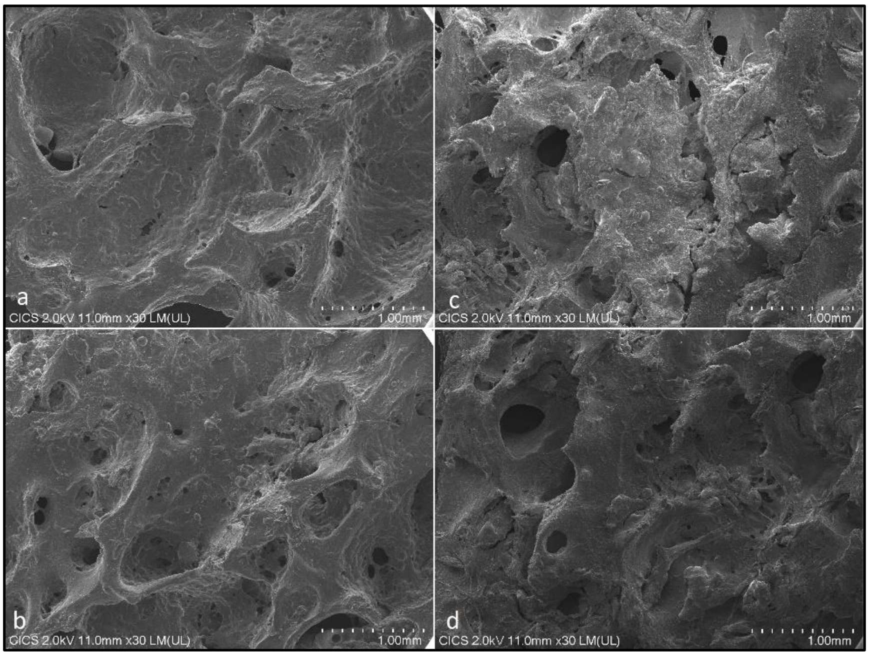

Figure 2 SEM images at low magnification (×30) of cancellous bone from the F bone (a and b) and scCO2 bone (c and d) groups.

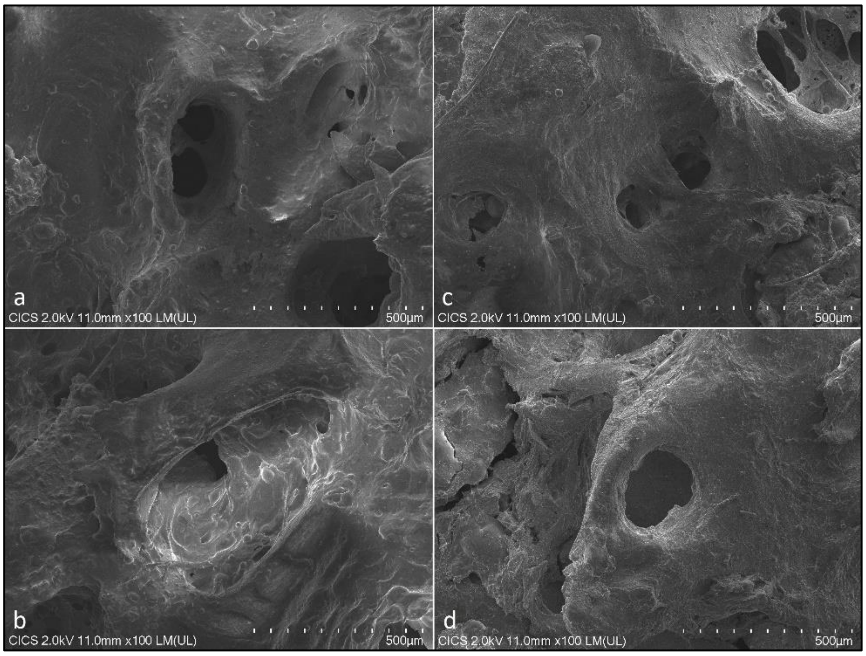

Figure 3 SEM images at medium magnification (×100) of cancellous bone from the F bone (a and b) and scCO2 bone (c and d) groups.

At high magnification (Fig. 4) the appearance of the two groups of bones was slightly different. The surface of the F bones was less rough than that of the scCO2 bones (Fig. 4a and b). In the scCO2 bone group, it had a multidirectional fibrillar appearance (Fig. 4c) and was rougher and grittier (Fig. 4d).

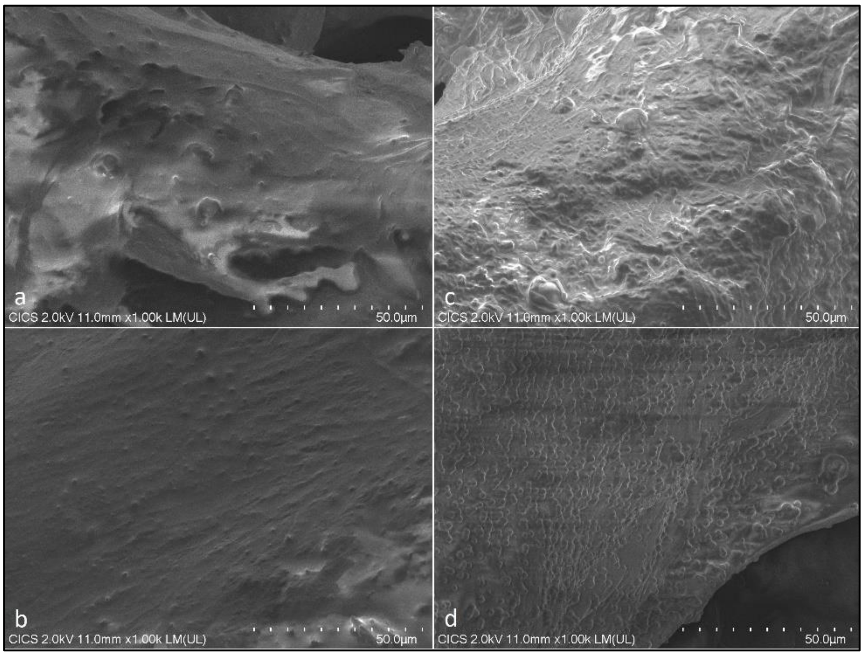

Figure 4 SEM images at high magnification (×1000) of cancellous bone from the F bone (a and b) and scCO2 bone (c and d) groups.

The correlation coefficient of the two sets of medullary cavity counts per image was high at 0.81. On average, 10.2 ± 3.3 and 9.1 ± 3.1 large cavities (>25 µm) (for the F bone and scCO2 bone groups, respectively) were found per image at ×100 magnification ($p = 0.4$). In the scCO2 bone group there were significantly more small cavities (<25 µm) (12.3 ± 2.3 versus 9.1 ± 3.1, $p = 0.02$).

4. Discussion

This comparative study evaluated the effect of scCO2 treatment after simple mechanical washing (without the addition of chemical co-solvent) on the mechanical and structural properties of human bone versus freezing at −80°C. The maintenance of the specific characteristics of native bone is essential for both the physical reconstruction of lost bone and for osseointegration. The combined analysis of all the variables measured supports the hypothesis that this treatment causes only slight changes in these properties for both cancellous and cortical bone.

Macroscopic mechanical tests showed that scCO2-treated bone was stiffer, stronger, and harder, but less deformable than frozen bone. These differences were significant in compression tests for cancellous bone, and in three-point bending tests for cortical bone. These findings were expected because bone is a biphasic material, composed of a fluid and a solid phase, which accounts for its viscoelastic behavior, particularly for cancellous tissue. Bone treatment leads to dehydration and stiffening owing to delipidation, with an increase in the modulus of elasticity [6,8]. Mechanical studies on cancellous bone are numerous and have yielded similar results [9–12]. However, direct comparisons of values are difficult because of large standard deviations due to the variability of the bones tested (different patients and anatomical locations). Conversely, few data are available on the effect of physicochemical treatment processes on cortical bone (apart from irradiation and freeze-drying) because they are generally not validated on this type of bone.

The mechanical results were supported by the histomorphometric analysis of bone structure, which found no significant difference between the two bone groups for any of the criteria. The cleaning treatment thus had no adverse effect on the bone volumetric density or spatial structure. The mean values of the variables measured corresponded to those of a non-osteoporotic adult population, in line with our recruitment [13,14].

The SEM study of the bone surface showed that medullary cavities were emptier and more numerous (small cavities less than 25 µm) in the scCO2 bone group than in the F bone group. Similarly, at high magnification, the surface in the scCO2 bone group was more fibrillous, which may correspond to collagenous organization. These findings can be explained by the cleaning effect of the simple mechanical wash associated with scCO2 treatment compared to cryopreserved bone. This confirms the findings of Rasch et al. [15], who had already reported similar results on chemical treatments of bone but also on the major effect of sonication on decellularization.

One of the limitations of this study arises from inter-individual variation in bone properties. Although this bias was controlled in part by directly comparing a fragment from the F bone and scCO2 bone group from the same patient (and the same anatomical area) for each of the measurements, there were large standard deviations in the results, which weakened the power of the study. Another limitation concerning the mechanical tests was that the tests performed were static and not cyclic loads like those received by a bone in real life. Concerning the histomorphometric analysis, a better micro-CT resolution (<100 µm) would have allowed a finer and more objective view of the three-dimensional characteristics of the bone [14] as a complement to the SEM. Finally, comparisons were made against frozen and not fresh bone, because this is the reference mode of preservation of allografts and the mechanical consequences of this preservation are negligible [16].

5. Conclusion

Human cortical or cancellous bone treated by a new process with no chemical solvent retains mechanical and structural properties close to those of bone frozen at −80°C. Work is now needed to validate the biological properties, in particular by evaluating bone osteoblastic recolonization (proliferation and differentiation).

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

No conflict of interest.

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