Evaluation comparative par ultrasons des propriétés d'allogreffes osseuses après traitement par différents procédés de viro-inactivation

Résumé de l'étude du Dr Laurent VASTEL publiée dans
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Introduction

Les procédés de traitement de l'os spongieux disponibles font appel à des technologies dont l'impact sur les propriétés mécaniques de l'os spongieux est mal connu. Ce travail a pour but de comparer quatre procédés par analyse ultrasonore d'échantillons appariés avant et après traitement de viro-inactivation. La vitesse de propagation des ultrasons à travers le réseau trabéculaire osseux est corrélée à l'élasticité du tissu osseux. En mesurant la variation de la vitesse due au traitement, on en déduit son influence sur l'élasticité du tissu osseux. Ce test étant non destructif, les résultats obtenus sont statistiquement significatifs.

Méthodologie

- ***Traitements comparés :***
  1. Procédé Supercrit® de BIOBank (Presles-en-Brie - France)
  2. Procédé Phoenix® de TBF (Mions - France)
  3. Procédé Tutoplast® de Tutogen (NeunKirchen - Allemagne)
  4. Procédé à l'Urée 6M (France)

- ***Matériel et méthode :***
  - Les échantillons sont des cubes d'os spongieux découpés au centre de têtes fémorales prélevées sur donneurs décédés. Les procédés n° 1, 2 et 3 ont été comparés au sein de la même étude[2]. Le procédé n° 4 a fait l'objet d'une étude spécifique[1]. Le protocole suivi est identique pour les 2 études et autorise donc la comparabilité des résultats.
  - Echantillonnage -> 28 échantillons appariés pour les procédés n° 1, 2 et 3
    18 échantillons appariés pour le procédé n° 4
  - Chaque échantillon du groupe est traité par un des quatre procédés.
  - La mesure de la vitesse de propagation des ultrasons est réalisée avant et après traitement. Chaque échantillon est ainsi comparé à lui-même, la mesure initiale correspondant à celle de l'os frais non traité.
  - Après traitement, une mesure de densité est réalisée pour le calcul du module d'élasticité E, selon la formule $\mathbf{E = \sigma . v^2}$ (σ est la densité apparente et v la vitesse des ultrasons)

Résultats

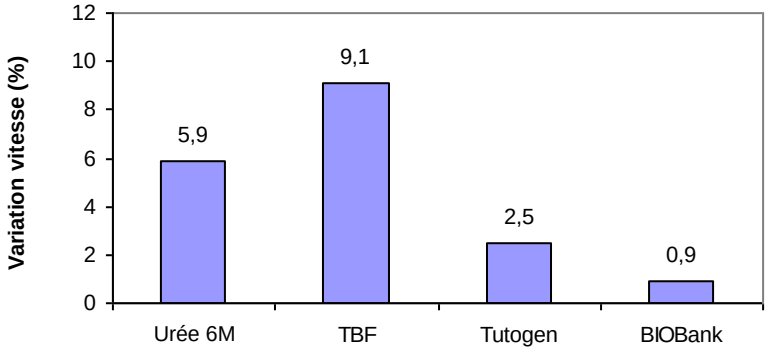
Selon les traitements de viro-inactivation, les propriétés biomécaniques de l'os spongieux peuvent être altérées à deux niveaux : architecture trabéculaire et densité osseuse.

La variation de la vitesse ultrasonore marque une altération de l'architecture trabéculaire du tissu osseux par le procédé. La diminution de la densité osseuse provient des produits chimiques dénaturants utilisés. Plus la densité osseuse diminue plus la variation du module d'élasticité augmente.

La variation du module d'élasticité, exprimée en pourcentage, permet de quantifier l'influence d'un procédé sur les propriétés biomécaniques du tissu osseux.

- ***Résultats biomécaniques comparés :***

Procédé mis en œuvre	Variation Vitesse (%)	Variation Elasticité (%)
Urée 6M (n=18)[1]	- 5.9	- 26.3
TBF (n=28)[2]	- 9.1	- 17.2
Tutogen (n=28)[2]	- 2.5	- 4.8
BIOBank (n=28)[2]	- 0.9	- 1.7



Les résultats démontrent que seul le procédé Supercrit® de BIOBank n'entraine pas d'altération significative des propriétés biomécaniques de l'os spongieux frais. A l'inverse, les autres procédés, notamment ceux de TBF et à l'Urée 6M, ont un impact significatif sur le module d'élasticité.

Discussion

L'utilisation de produits chimiques dénaturants des protéines, nécessaires à l'obtention d'une viro-inactivation efficace, entrainent des modifications importantes de la résistance de l'os trabéculaire par leur action sur le collagène osseux.

Le procédé à l'urée 6M et le procédé de TBF qui utilise de l'hypochlorite de sodium, sont particulièrement agressifs. Le procédé de Tutogen, utilisant principalement de l'acétone, donne lieu à une modification modérée de l'élasticité. Le procédé Supercrit® de BIOBank utilise le CO_2 supercritique, connu pour ses propriétés délipidantes mais aussi pour sa capacité à respecter les protéines. Ces propriétés se traduisent par une meilleure préservation du collagène osseux.

Conclusion

Il ressort de cette étude que le procédé Supercrit® est le traitement qui apparaît le plus approprié pour préserver les qualités structurales et architecturales de l'os natif. Le chirurgien se doit de prendre en compte l'effet des procédés dans le choix de ses allogreffes pour les reconstructions orthopédiques qui requièrent un haut niveau de résistance mécanique.

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[2] Vastel L., Masse C., Mesnil P., Crozier E., Padilla F., Laugier P., Mitton D., Courpied J. P. Comparative ultrasound evaluation of human trabecular bone graft properties after treatment with different sterilization procedures. J Biomed Mater res Part B: Appl Biomater 2009 Jul ; 90B(1): 430-7

Comparative Ultrasound Evaluation of Human Trabecular Bone Graft Properties After Treatment With Different Sterilization Procedures

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Abstract: New sterilization methods for human bone are likely to affect the mechanical properties of human cancellous grafts. These mechanical properties dictate the short- and mid-term results of the orthopedic procedure. The aim of this study was to compare the effects on bone mechanical properties, as assessed by ultrasound velocity, of different sterilization methods used under similar conditions: bleach and sublimation, humid heat, successive baths of physiological saline with osmotic detersion, and CO_2 in the supercritical phase. Alterations in mechanical properties were small with CO_2 (velocity change: −2%) and humid heat (−2.5%). Osmotic detersion had a significant but moderate effect (−4.7%). The −9% change with the protocol involving bleach suggested a greater than 30% decrease in load to failure, based on earlier studies. Gamma irradiation of defatted trabecular allografts, in a dose of 10 or 25 KGy, produced no significant changes in ultrasound velocity. Powerful protein denaturants used in sterilization protocols substantially alter the mechanical resistance of the grafts, which may jeopardize the orthopedic procedure. © 2009 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 90B: 430–437, 2009

Keywords: allograft; sterilization; trabecular bone; ultrasound velocity

INTRODUCTION

Cancellous bone allografting is now widely used in orthopedic surgery, especially for hip revision, tumor, and trauma. Bone transplants are usually obtained from patients undergoing hip arthroplasty and assessed for safety within a tissue repository. The emergence of new transmissible diseases (such as bovine spongiform, encephalopathy, and hepatitis) has generated considerable concern about bone graft safety over the last decade. Safety concerns are greatest when bone grafts are used to treat nonlife threatening diseases in young patients.

To improve the safety of allogeneic bone grafts, new sterilization methods have been developed,[1–5] and previous methods have been implemented more widely.[6–10] Currently, the most commonly used methods consist of treatment with chemical agents (NaOH, 6*M* urea, or hypochlorite) to denature prions followed by beta or gamma irradiation[7–9] to kill bacteria and viruses. These methods are more effective when performed after bone marrow removal, which also accelerates subsequent osteointegration of the graft.[11]

The mechanical resistance of treated grafts has a major influence on the strength of the orthopedic construction prior to osteointegration. Comparative data on the mechanical resistance of treated and untreated bone grafts are needed to guide surgical decisions. In a recent study,[12] 6*M* urea treatment induced a significant loss of mechanical resistance with a decrease in ultrasound velocity and abnormalities in conventional mechanical tests. Ultrasound velocity is directly correlated to the load to failure.[12–17] As with cortical bone, cancellous bone from cadavers has been investigated for relationships between ultrasound velocity and mechanical properties. Strong correlations were found between ultrasound parameters (attenuation and velocity) and mechanical properties (Young's modulus or ultimate strength). These correlations were noted with both native

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bone and defatted bone,[16] and a study showed that the intrinsic mechanical properties were not affected by the presence or absence of fat *in situ*.[18] Bone mineral density and ultrasound velocity along a single axis have nearly similar ability for predicting site-matched elastic modulus and bone strength.[13] This good performance of ultrasound velocity for predicting bone strength can be reasonably assumed to extend to bone graft material.

Nevertheless, marked differences exist between ultrasound velocity measurements and conventional mechanical tests. Vastel et al.[12] found that load to failure decreased by about 35% when ultrasound velocity decreased by 6.2%; thus, an about 2.5% decrease in ultrasound velocity was associated with an about 10% decrease in load to failure.

In hip arthroplasty involving acetabular revision, the orthopedic construction is exposed to stresses of 1–3 MPa[19–21] with load peaks to 8.8 MPa.[21] Before osteointegration occurs, the success of the reconstruction depends on the initial strength of the implanted material, which is governed by the strength of the allograft, because bone is the weakest link at this time. Objective comparisons of bone-graft treatments are useful to practitioners, especially as loads applied on the hip *in vivo* are only slightly less than loads to failure measured on fresh bone.[12–14]

The aim of this study was to evaluate the effects on mechanical resistance of treatments that are widely used to sterilize human bone grafts. We measured ultrasound velocity to evaluate mechanical resistance.

MATERIALS AND METHODS

Source of Bone

Samples were obtained from femoral heads removed during multiorgan collection, which complied with the requirements of the French Transplant Administration. The heads were from 14 donors who had no known hip disease, nine men and five women whose ages ranged from 23 to 58 years.

Sample Preservation

Between treatment steps, the samples were placed in isotonic saline and refrozen at −40°C.

Sample Preparation

Samples were prepared using previously described methods.[12] Briefly, the femoral heads were pared using an oscillating saw, along the main load bearing axis during life. Then, one or two 9-mm thick slices including the central zone of the femoral head were cut (Figure 1) using a low-speed saw, with continuous saline irrigation. Four parallelepipeds were then obtained from the central slices, which were oriented and numbered. Each sample was labeled using methylene blue to show its position in life relative to the main compressive loads applied to the femoral head.

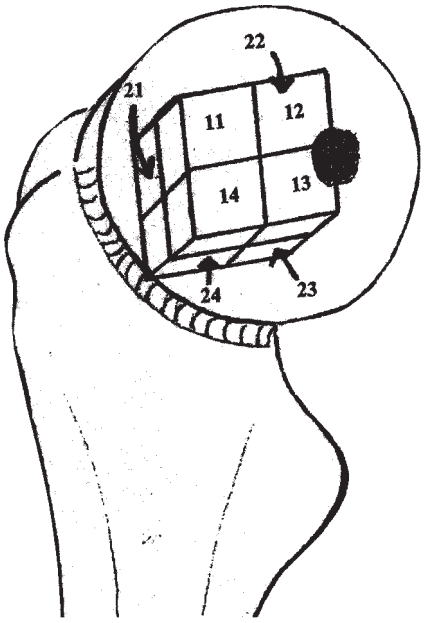

Figure 1. Cutting and numbering of femoral head samples.

Sample dimensions were then measured relative to the mechanical axis, using digital calipers, as an additional control test relatively to the main load bearing axis. We prepared 192 samples from the 14 donors.

Ultrasound Velocity Measurement

Ultrasound velocity was measured using two pairs of unfocused transducers with center frequencies of 60 kHz (model R6, Physical Acoustics Corp, Princeton Jct, NJ) and 2.25 MHz (model WS 75-2, Ultran, State College, PA), respectively. The system consisted of a pulser/receiver device having a frequency bandwidth of 1 kHz–35 MHz (Panametrics model 5052 PR, Waltham, MA), a preamplifier (Data precision D1000 Dual Preamp, Analogic, Peabody, MA), and a 60-MHz digital oscilloscope (TDS 210, Tektronics, Beaverton, OR) connected to a laptop computer equipped with a Tekvisa acquisition module (Tektronics, Beaverton, OR). Measurements were conducted through opposite parallel faces of the sample. The signal measured with no specimen between the transducers was taken to indicate the amount of noise. Ultrasound velocity was measured using a threshold value greater than 10-fold the amount of noise, by dividing the transit time through the sample by the thickness of the sample. Measurements were obtained for each frequency along the three orthogonal axes, that is the direction of the main compressive loads in life and the two axes perpendicular to that direction.

Apparent Density (σ) and Modulus of Elasticity (Ei). To evaluate the modulus of elasticity from low-frequency measurements, we determined the apparent density of each sample before and after application of each of the studied

TABLE I. Details of Each of the Four Sterilization Procedures Tested in Our Study

	TBF®	Moist Heat	Tutoplast®	Supercrit®
Step 1	Sodium azide (12 h), ethanol-chloroform (19 h), H_2O_2 (19 h), Atm. pressure, delipidation-sterilization	Humid heat, 125°C 20 min, sterilization	Successive baths with isotonic saline, H_2O_2, and acetone (>48 h), Atm. pressure, delipidation	CO_2 in supercritical phase, 26 Mpa 50°C, delipidation-sterilization
Step 2	Hypochlorite (2 h), Atm. pressure, Sterilization ag. Prion		Rinsing drying, Atm. pressure	H_2O_2, NaOH, ethanol, Atm. pressure, delipidation sterilization
Step 3	Sublimation, drying		18 KGy, sterilization after packaging	Drying, Atm pressure
Step 4	25 KGy, Sterilization after packaging			25 KGy, sterilization after packaging

treatments. A balance (accuracy 0.01 g) was used to measure the mass of each treated dehydrated specimen. The external volume of each specimen was computed from the lengths of the three dimensions of the parallelepiped, which were measured using micrometric calipers. The modulus of elasticity was computed using the equation $Ei = \sigma v^2 i$, where Ei is the modulus of elasticity, σ the apparent density, and v ultrasound velocity.[13,14]

Measurement Conditions

Samples were defrosted 16 h before the measurements, at 4°C. Therefore, the number of defrosts was identical for all samples. For each test session, 40 samples were defrosted. All test sessions were conducted in the same room, at a temperature close to 20°C with fluctuations no greater than ±2°C, by the same operator.

Repeatability measurements were carried out on various standard materials (aluminum, stainless steel, copper, and brass) then on four bone samples. The measurements of the standard materials at the beginning of each test session allowed us to validate the measurement conditions and to check that all devices were working properly. For the repeatability measurements on samples, each of the four samples was tested six times at both frequencies along the three axes. We conducted repeatability measurements with two hydration methods as follows: immersion of the sample in isotonic saline then placement of the sample on the receiver, the mean time between removal from saline to data acquisition being 30 s; or immersion of the sample in isotonic saline then placement of the sample on paper towel for 1 s. The mean time between removal from saline to data acquisition was 60 s. The mean coefficient of variation (%) of six measurements on each of the four samples was four times lower using the procedure that did not involve paper towel. This procedure was therefore selected for the study. Its coefficients of variation were 0.95% and 0.73% for low and high frequencies, respectively, indicating excellent reproducibility.

In addition, results of repeatability measurements allow us to determine the least significant change as reported by Gluer[22]: $L = 2\sqrt{2} \times \sigma$.

The least significant change for this protocol was 2.7% for low frequency and 1.1% for high frequency sound, respectively. Differences below this value were considered as nonsignificant.

Graft-Processing Treatments

The study involved two parts, one on 112 samples and the other on 80 remaining samples.

In the first part of the study, four groups each comprising 28 samples were subjected to the following treatments (Table I).

TBF® (TBF Genie Tissulaire, Mions, France). Samples were soaked successively in sodium azide (12 h), ethanol/chloroform (19 h), and hydrogen peroxide (19 h) to remove fat and to clean cancellous bone; the samples were then neutralized, soaked in hypochlorite for 2 h to eradicate prions, rinsed, lyophilized by sublimation, packaged, and exposed to 25 KGy gamma radiation to kill bacteria and viruses.

Moist Heat. Samples were exposed to humid heat at 125°C for 20 min. This procedure, which does not include cleaning, chemical treatment, or irradiation, is used by the bone bank in Toulouse, France.

Tutoplast® (Tutogen®, NeunKirchen, Germany). Samples were soaked in successive water baths then in isotonic saline, hydrogen peroxide, and acetone; the samples were then rinsed, dried, packaged, and exposed to 18 KGy gamma radiation.

Supercrit® (Biobank®, Paris, France). It involves treatment with supercritical CO_2 (26 Mpa, 50°C) for bone cleaning and lipid extraction, as described by Fages et al.[23]; successive baths in hydrogen peroxide (H_2O_2,, 35%), sodium hydroxide (NaOH, 4%), and ethanol; drying; packaging; and exposure to 25 KGy of gamma radiation.

In the second part of the study, three groups each comprising 20 samples were tested after each of the three steps of the Supercrit® procedure used above: supercritical CO_2

TABLE II. Comparison of Speed of Sound Before Sterilization

Mean (Standard deviation) ms^{-1}	LF Main Axis	LF Ortho1	LF Ortho2	HF Main Axis	HF Ortho1	HF Ortho2
TBF® ($n = 28$)	2266 (140)	2118 (161)	2138 (188)	2669 (137)	2465 (162)	2527 (213)
Moist heat ($n = 28$)	2284 (130)	2086 (171)	2126 (132)	2691 (136)	2468 (211)	2541 (180)
Tutoplast® ($n = 28$)	2320 (156)	2120 (141)	2101 (130)	2720 (152)	2502 (171)	2491 (168)
Supercrit® ($n = 28$)	2329 (128)	2105 (153)	2133 (152)	2727 (124)	2492 (182)	2537 (149)
Supercritical CO_2 only ($n = 20$)	2341 (80)	2165 (134)	2150 (186)	2722 (89)	2498 (169)	2538 (199)
Sup. CO_2 + chemicals ($n = 20$)	2343 (95)	2137 (164)	2189 (177)	2728 (82)	2472 (173)	2544 (189)
Supercrit® ($n = 20$)	2342 (97)	2163 (122)	2194 (182)	2721 (108)	2505 (122)	2567 (214)
Modif. Supercrit® ($n = 20$) (10 KGy instead of 25 KGy)	2343 (101)	2102 (127)	2152 (206)	2735 (103)	2451 (150)	2511 (244)

The four sterilization procedures (TBF®, Tutoplast®, moist heat, and Supercrit®) are detailed in Table I. "Supercritical CO_2" refers to the first step of the Supercrit® procedure and "supercritical CO_2 plus chemicals" to the first two steps.

LF, low frequency; HF, high frequency; ortho 1 and ortho 2, the two axes perpendicular to the main loading axis in vivo.

only, supercritical CO_2 followed by chemicals, and full procedure (supercritical CO_2, chemicals, and 25 KGy gamma radiation). A fourth group of 20 samples was tested after the first two steps of the Supercrit® procedure followed by exposure to 10 KGy gamma radiation (instead of 25 KGy).

Statistical Analysis

All statistical tests were carried out using R software (R Foundation for statistical computing, Vienna, Austria). The Shapiro-Wilk normality test consistently showed heterogeneity of the data across axes, frequencies, and treatments. We therefore used nonparametric tests to ensure that the same system could be used for all comparisons. For each frequency and each axis, the Friedman test (one-way analysis of variance by ranks) was used to compare the similarity of multiple groups; p values less than 0.05 were considered significant. When this test revealed a treatment effect, the Wilcoxon test was used to compare paired groups.

Comparison of the Supercrit® Groups in the Two Parts of the Study

Two groups of samples received the Supercrit® procedure: the Supercrit® group in the first part of the study and the group that received all three steps of the procedure (with 25 KGy) in the second part of the study. To check the consistency of our findings, we compared the results in these two groups.

RESULTS

Measurements Before Treatment

Group Similarity.

First Part of the Study. Results of measurements done before treatment are reported in Table II.

The Friedman test showed a difference across groups ($p = 0.0117$). The Wilcoxon pairwise test detected a difference between the TBF® group and the Supercrit® group, with high-frequency sound only. The maximum difference for high-frequency sound was 2.2% along the main axis.

Second Part of the Study. The Friedman test showed that the four groups were similar before treatment (supercritical CO_2 only, supercritical CO_2 and chemicals, all three steps with 25 KGy gamma radiation, and all three steps with 10 KGy instead of 25 KGy) ($p = 0.2105$).

Measurements After Treatment

First Part of the Study. comparisons of the four treatment procedures. Table III reports the relative velocity changes induced by treatments. The TBF® procedure induced a statistically significant ($p < 0.05$) change that ranged from 7.8% to 10.2% according to the axis and frequency. The Tutoplast® procedure induced a smaller but still significant change ($p < 0.05$) ranging from 2.5% to 5.7%. The treatment-induced change differed significantly between TBF® and Tutoplast® along all axes for low-frequency sound. Both moist heat and Supercrit® induced nonsignificant changes at low frequency measurement and small but significant changes at high frequency ($p < 0.05$), which ranged from 1.3% to 2.2%.

Modulus of elasticity values before and after the treatments are reported in Table IV.

It is important to underline that only one statically significant change is reported for TBF procedure ($p < 0.05$). None of the other treatments had showed a significant effect on the modulus of elasticity, for measurements with high and low frequency.

Second Part of the Study: Supercrit® Procedure Steps. Table V reports the impact of adding each step of the Supercrit® procedure and of reducing the gamma radiation dose from 25 to 10 KGy. Each step produced small but statistically significance changes; the mean maximum change was 3.2% along the main loading axis and 5% along the other two axes. The Friedman tests confirmed this observation, showing that the differences between treatment steps were no greater than 2%. Reducing the gamma radiation dose from 25 to 10 KGy produced a sig-

TABLE III. Relative Changes in Speed of Sound (in %) Induced by Each Sterilization Procedure

		Variation (%) LF Main Axis	Variation (%) LF Ortho1	Variation (%) LF Ortho2	Variation (%) HF Main Axis	Variation (%) HF Ortho1	Variation (%) HF Ortho2
TBF®	Mean (St. dev.)	9.1** (5.0)	9.4** (4.2)	10.2** (4.8)	9.0** (4.6)	7.8** (4.0)	9.1** (4.5)
$n = 28$	Min/Max	−1.5/19.3	0.3/16.2	0.8/21.9	−1.3/19.8	−0.8/15.7	−0.8/15.7
Moist heat	Mean (St. dev.)	0.7* (2.3)	1.5* (4.2)	0.6* (3.1)	2.1** (1.3)	1.3** (3.4)	1.8** (1.1)
$n = 28$	Min/Max	−3.6/5.5	−12.5/10.1	−10.3/6.6	−0.6/6.5	−9.8/9.9	−1.3/4.5
Tutoplast®	Mean (St. dev.)	2.5* (2.6)	2.6* (4.7)	5.2** (5.4)	4.7** (2.0)	4.0 ** (2.7)	4.0** (4.2)
$n = 28$	Min/Max	−7.6/6.7	−8.9/9.1	−4.6/24.3	0.7/10.5	−3.9/8.9	−10.7/11
Supercrit®	Mean (St. dev)	0.9* (2.3)	1.0* (2.6)	0.6* (2.3)	1.8** (1.8)	1.8** (1.6)	2.2** (1.7)
$n = 28$	Min/Max	−4.2/4.5	−4/7.1	−5.4/5.2	−4.3/4.6	−2.9/6.0	−1.1/6.9

*, variation below the least significant change.
**, statistically significant difference by the Wilcoxon test ($p < 0.05$).

nificant difference with high-frequency sound only, in keeping with the greater measurement accuracy with high-frequency than low-frequency sound. The results indicated less mechanical alteration with the full procedure than with the incomplete procedure, suggesting that gamma radiation delivered to dry bone, in the doses used for our study, may in some cases improve the mechanical properties of bone. Nevertheless, the different steps of the Supercrit® procedure exerted similar effects on ultrasound velocity and modulus of elasticity. Final irradiation of dried bone had little effect on the mechanical properties of the samples.

DISCUSSION

Measurement Validity

The ultrasound velocity values obtained in our study are consistent with previously published data (Ashman and Rho,[14] Vastel et al.[12]), being only slightly higher, probably because of the younger age of our donors (mean, 46 years). Indeed, the mechanical resistance of cancellous bone decreases with age.[24,25] Other sources of discrepancy across studies may include differences in the algorithms used to determine time-of-flight through the sample.

The repeatability tests allowed us to choose the optimal measurement conditions and to control measurement reliability. We compared ultrasound velocity at baseline and after processing, to eliminate the effect of confounding factors, such as bone density variations across individuals or across bone sites in a given individual (Weaver et al.[24]), particularly within the femoral head and neck (Brown and Ferguson[26]). Our procedure provided direct nondestructive measurements of the effects of treatments on intrinsic material properties and cancellous bone structure. The similarity between results in the two identically treated groups (Supercrit® group in the first part of the study and group treated with all three steps of the Supercrit® procedure in the second part of the study) confirms the good reproducibility of our measurements. The anisotropy measurements allowed us to validate the conditions of sample extraction, preservation, and preparation. Previous studies[26,27] have established that acoustic anisotropy reflects mechanical anisotropy between the axis of greatest loading and the two perpendicular axes. Finally, the correlation between the values measured at high and low frequencies (Figure 2) further supports the validity of our results. Thus, the ultrasound velocity changes found in our study can be ascribed to the treatments used on the samples.

The good reproducibility of our measurement protocol allowed us to compare several widely used procedures for bone-graft sterilization. In addition, the study methods were similar to those in an earlier study by our group,[12] allowing us to compare the results (Figure 2).

Comments

The use of powerful chemicals to achieve protein denaturation causes structural changes in cancellous bone proteins,[28] probably including type I collagen, and therefore induces major changes in the mechanical resistance of the graft. These changes are well illustrated by our results with the TBF® procedure, which includes bleach. Similarly, an earlier study found marked adverse effects of 6*M* urea treatment on mechanical properties of bone. Chemical solvents or CO_2 at the supercritical phase, which ensures complete removal of the bone marrow *in situ*, resulted in significant but small alterations in mechanical properties, in agreement with a previous study.[3] Procedures that include complete bone marrow removal may accelerate osteointegration.[11] The physical treatments used in our study (moist heat and 10 or 25 KGy of gamma radiation) caused small alterations in mechanical properties. If it is well-known than physical treatments also denatures proteins, studies had shown that effects of protein-denaturing treatments depends on the dose, as reported with gamma radiation.[10,12] Regarding the effects of heat, fibrillar collagen I proteins are probably less sensitive to heat than structural prions proteins. This may explain why moist heat as used in our study had limited effects on mechanical bone properties.

Because heat treatment is effective and inexpensive, it may be an interesting tool; the scant amount of data on bone responses to heat needs to be supplemented by further studies. It should be borne in mind that gamma irradiation of nondefatted bone has been reported to induce the release

TABLE IV. Relative Changes in Elasticity Modulus (in %) Induced by Each Sterilization Procedure

	Part I	Variation E (%)Main Axe	Variation E (%) Ortho1	Variation E (%) Ortho2	Part II	Variation E (%) Main Axe	Variation E (%) Ortho1	Variation E (%) Ortho2
Mean (Std Dev.)	TBF® (n = 28)	17.2* (9,1)	18.6* (8,6)	18.2* (9,3)	SC CO^2 (n = 20)	3.5 (2,9)	9.6 (5,2)	9.1 (12,8)
Min/Max		−3.0/34.9	8.6/39.5	−0.7/39		−0.8/8.4	0.9/18.7	−28.7/29.7
Mean (Std Dev.)	Moist heat (n = 28)	1.3 (4.7)	0.9 (11.2)	0.6 (6.6)	SC CO^2 + Chemicals (N = 20)	5.9 (5.4)	7.5 (4.8)	5.1 (4.6)
Min/Max		−7.2/10.7	−35.3/19.1	−21.8/12.8		−4.0/20.8	−0.8/19.2	−6.7/14.1
Mean (Std Dev.)	Tutoplast® (n = 28)	4.8 (5,2)	4.7 (9,1)	7.8 (13,9)	Full proc. Supercrit (n = 20)	5.0 (4,6)	3.3 (5,4)	4.9 (6,3)
Min/Max		−15.7/12.9	−18.5/17.5	−40.1/42.7		−2.3/13.1	−9.8/10.7	−9.3/14.4
Mean (Std Dev.)	Supercrit® (N = 28)	1.7 (4,5)	1.6 (5,3)	1.3 (4,5)	Modified Supercrit (10 KGy) (n = 20)	5.7 (4,4)	4.7(10,2).	4.5 (8,9)
Min/Max		−8.5/8.8	−8.4/13.7	−11.0/10.0		−2.9 /14.8	−20.9/25.9	−24.0/25.7

*, variation statistically significant using the Wilcoxon test ($p < 0.05$).

TABLE V. Relative Variation in Velocities (in %) After Each of the Three Steps of the Supercrit® Procedure (Supercritical CO_2, Chemicals, and 25 KGy Gamma Radiation)

		Variation (%) LF Main Axis	Variation (%) LF Ortho1	Variation (%) LF Ortho2	Variation (%) HF Main Axis	Variation (%) HF Ortho1	Variation (%) HF Ortho2
Step 1: Sup. Crit. CO_2 n = 20	Mean (St. dev.)	1.7* (1.4)	5.0 (2.7)	4.9 (6.5)	1.4 (1.6)	3.5 (2.2)	2.3 (4.3)
	Min/Max	−0.4/4.3	0.5/9.8	−13.4/16.2	−1.9/6.2	0.6/10.0	−12.8/9.0
Sup. Crit CO_2 + chemicals n = 20	Mean (St. dev.)	3.0 (2.8)	3.8 (2.5)	2.6* (2.3)	3.2 (1.6)	3.0 (1.8)	2.4 (1.6)
	Min/Max	−2.0/11.0	−0.4/10.1	−3.3/7.3	−0.4/6	−1.4/7.0	−1.5/4.9
Full Supercrit® procedure n = 20	Mean (St. dev.)	2.5* (2.3)	1.8* (2.6)	2.5* (3.2)	1.8 (1.8)	1.2 (1.9)	1.9 (1.5)
	Min/Max	−1.1/6.8	−4.8/5.5	−4.5/7.5	−2.0/5.1	−1.1/5.6	−0.3/6.0
Variant of Supercrit® (10 instead of 25 KGy) n = 20	Mean (St. dev.)	3.0 (2.3)	2.7* (5.2)	2.3* (4.5)	3.0 (1.4)	2.7 (4.6)	2.2 (5.2)
	Min/Max	−1.5/7.7	−10.0/13.9	−11.3/13.8	0.1/5.5	−11.0/16.4	−14.8/15.0

The asterisks indicate variations smaller than the least significant change. All others results were statistically significant using Wilcoxon test ($p < 0.05$).
Ortho 1 and ortho 2, the two axes perpendicular to the main loading axis in vivo.
LF indicates low frequency and HF high frequency sound waves.
E, modulus of elasticity.

of toxic compounds.[29] The two defatting procedures used in our study differed in their effects on the mechanical properties of cancellous bone: CO_2 at the supercritical phase seemed less aggressive than acetone (Figure 3).

Our results established that sterilization procedures substantially affected the mechanical properties of cancellous bone. Their impact should be kept in mind, and procedures associated with the greatest mechanical alterations should be avoided for extensive reconstructions, whose success rate is governed in part by graft strength.

The alteration in bone mechanical properties found after treatment with 6*M* urea[12] did not distinguish the damage due to molar urea from the damage caused by irradiation. In our study, gamma irradiation with 10 or 25 KGy did not significantly modify ultrasound velocity compared with nonirradiated treated bone. Thus, irradiation of dry bone does not seem to induce meaningful changes in mechanical properties. In the second part of our study, gamma radiation (compared with the first two steps of the Supercrit® procedure) improves the modulus of elasticity along most of the axes and with both sound frequencies (Table V). Conceivably, irradiation of dry bone may increase trabecular stiffness, thereby apparently improving ultrasound wave transmission. Studies specifically designed to investigate this hypothesis would be of interest. However, the effect of gamma radiation was small and probably of little clinical relevance.

CONCLUSIONS

This study, which complements our previous study conducted using the same method, allowed us to compare the effects of various sterilization procedures on the mechanical properties of cancellous bone. Powerful chemicals used to achieve protein denaturation produced marked alterations in mechanical properties, indicating adverse effects on cancellous bone structure and probably on type I collagen. This effect of strong chemicals should be borne in mind when choosing graft material for orthopedic reconstructions that require a high level of mechanical resistance.

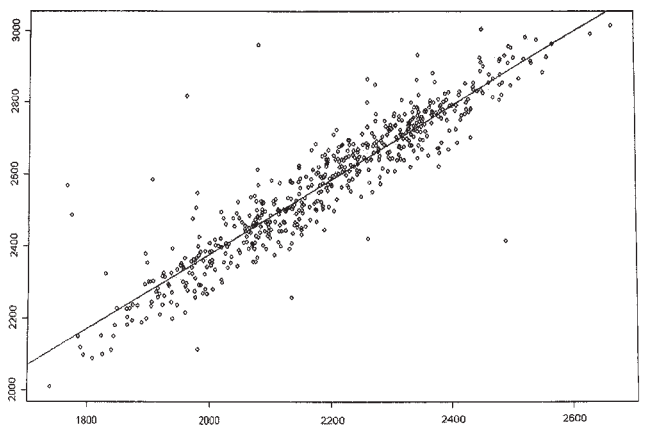

Figure 2. High frequency (HF) versus low frequency (LF) speed of sound (SOS) Horizontal Axis: LF SOS (ms^{-1}) Vertical axis: HF SOS (ms^{-1}).

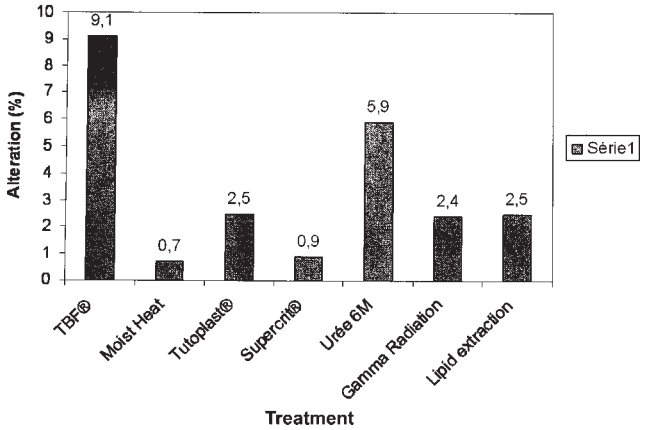

Figure 3. Results obtained at low frequency along the axis of usual main load in this study and Ref. 12.

Moderate but significant alterations in cancellous bone mechanical properties were noted after physical treatments (heat or gamma irradiation). No detectable effect was observed after final irradiation of dry treated bone.

A smaller but significant alteration in mechanical properties was noted with protocols, which used only bone-marrow cleaning agents. In this study, supercritical CO_2 had the smallest effects on ultrasound velocity, sometimes producing only barely detectable changes.

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Effect of different sterilization processing methods on the mechanical properties of human cancellous bone allografts

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Abstract

Use of new sterilization methods applied to human bone is likely to affect both the mechanical and biological properties of human cancellous grafts. The mechanical properties of the transplanted bone inevitably determine the short- and mid-term results of the orthopedic procedure performed. The aim of this study was to compare, under similar conditions, the mechanical effects of gamma irradiation, lipid extraction, and treatment with 6 M urea on trabecular bone samples, through conventional mechanical tests and measurement of the ultrasound wave propagation rate. Deteriorations measured for gamma irradiation and lipid extraction were low: 2.4% and 2.5%, respectively, for ultrasound propagation wave measurements. They were clearly significant for protocol including 6 M urea, corresponding to a loss of 30% in values measured in the control sample for the stress to failure, inciting prudence when grafted bone is used for support in orthopedic assembly. High consistency in the results obtained between travel time of the ultrasound wave, easily done, and measurement of stress to failure through conventional tests, favor the use of ultrasound protocol, described as a quality test performed on bone grafts in the tissue bank before distribution and implantation.

Keywords: Trabecular bone; Allografts; Sterilization methods; Mechanical properties

1. Introduction

The use of human cancellous bone grafts is now routine practice in orthopedic surgery, especially in hip revision and tumor or trauma surgery. Transplanted bone is obtained, in almost all cases from patients in whom the femoral head was collected during total hip arthroplasty, and is implanted after a safety of use assessment, performed within a tissue bank.

As a result of the occurrence of new transmissible diseases (bovine spongiform encephalopathy, hepatitis, etc.), concerns regarding safety of bone grafts have increased enormously over the last few years, in particular in cases of non-vital procedures often involving young patients.

The need to maintain maximum safety has resulted in the use of new sterilization methods [1–4] applied to human bone, and in more systematic use of some methods previously known for several years [5–7]. Currently, the most commonly used methods are irradiation procedures (beta and gamma) [8], for bactericidal and virucidal purposes, now used in some processing methods with chemical agents (NaOH or 6 M urea), with a view to denaturing unconventional transmissible protein agents (PRION).

These processing methods may follow lipid extraction (cleaning of non-mineralized tissue and excision of the bone marrow), thus enabling optimization of these procedures; lipid extraction according to some authors [9] is reputed to promote the process of subsequent osteointegration of the bone graft. Such processing methods are likely to affect both the mechanical and biological [2] properties of the trabecular bone.

However, in some cases, the mechanical properties of the transplanted bone graft inevitably determine the

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short- and mid-term results of the procedure performed. Thus, the aim of this study was to compare the effect of different sterilization processing methods, currently used in routine practice, on the mechanical properties of trabecular bone through conventional mechanical tests and measurement of the ultrasound wave propagation rate. The treatments studied were: gamma irradiation, mechanical lipid extraction, and treatment with 6 M urea.

2. Material and methods

2.1. Source of the test bone

Eighteen femoral heads were collected from 14 cadavers of 19–60 years of age, free from any known disease of the hip, in the context of multiorgan collection. Femoral heads were frozen immediately after excision. They were packaged without any additives in sterile, airtight, triple water-resistant plastic package and stored at −80°C until use.

2.2. Preparation of test samples

Considering interindividual variations in mechanical properties, matched comparisons only concerned samples from a same femoral head [10,11].

Taking into account the mechanical property distributions in the cancellous bone of the femoral head [12,13], matched samples were prepared from a restricted central area of the head, in which homogeneous mechanical properties could be supposed in native bone (Fig. 1). In this area, and in each femoral head, four cubes of identical volume were prepared, 9 mm thick. Ancillary equipment enabled obtention of strictly parallel 2×2 cutting planes. Seventy-two samples were obtained consisting in 18 groups of 4 samples.

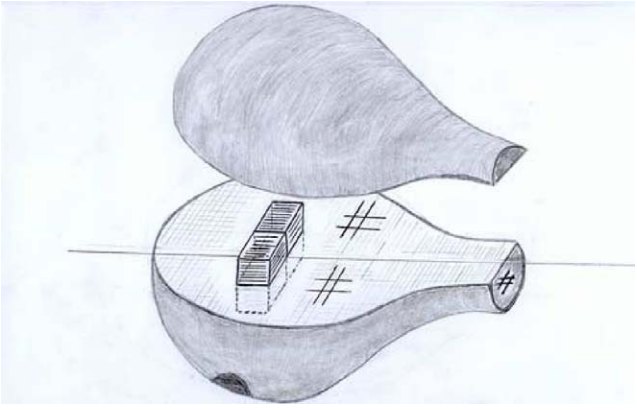

Fig. 1. Samples preparation, cut from a restricted central area of the femoral head.

2.3. Processing methods applied

In each group of 4 samples from a given femoral head, the samples underwent one of the following processing methods:

The control sample did not undergo any additional processing and was returned to the freezer at –80°C, in a test tube with physiological saline.

Lipid extraction: The samples involved were cleaned by pressure irrigation with physiological saline (90 bars) and were then immersed in a solution of pure acetone (50 ml) for 17 h in two successive baths. Then, the samples were returned in a test tube of physiological saline to the freezer.

Processing with 6 M urea: These samples underwent the different steps of lipid extraction as described above. They were then rinsed with pure water and immersed successively in a detergent solution and in 6 M urea solutions for a total of 2×3 h. After rinsing with pure water they were finally dried under a hot (37°C) air stream for 12 h. Before the first tests they were rehydrated in physiological saline during 3 h, and after the tests they were kept in physiological saline at −80°C.

γ-Irradiation: The samples involved were defrosted in the test tube and then irradiated with a dose of 30 kGy (from 29.3 to 36.3). They were then refrozen at −80°C.

2.4. A circular permutation between samples and treatments was undertaken

γ-Irradiation was attributed to sample A in the first group, B in the second, C in the third, etc.

In each group of 4 samples, the 4 treatments coexisted, and comparisons were realized between treated samples and the control sample of the group.

2.5. Measurement of the ultrasound wave propagation rate

The conduction rate was calculated, based on measurement of the ultrasound wave travel time within each sample. Each sample was directed to its reference anatomical orientation, in order to undergo testing aligned to the axis of transmission of body load stress in the living subject. Its thickness was measured using a micrometer (accuracy: 10 μm). A Sofranel® apparatus, model 5052 UA, operating in transmission mode, with a distinct transmitter and receiver, at a 2.25-MHz frequency, generated ultrasounds.

Measurement of transmission time was obtained after parameterizing the apparatus with a 5-mm thick Plexiglas puck.

These measurements were obtained after gradual and complete defreezing of the sample (3 h at room temperature). The sample was then kept moist during the different handling procedures. Measurement of the

transmission time in the different samples was carried out before and after processing.

Measurements before processing allowed us to evaluate, by a non-destructive procedure, the potential variations between test cubes from a given group of 4 samples from a same femoral head, and to validate the model selected.

The measurements obtained after processing allowed us to perform, for each sample, a matched comparison of conduction rate before and after processing.

The samples were then returned to the test tubes filled with physiological saline and refrozen at −80°C.

2.6. Mechanical tests

The machine used was a screw-filled tensile compression machine (Wolpert®). Each sample was deposited on the plate of the machine according to its anatomical orientation. Compression was applied at a rate of 2 mm per minute, with the mobile plate beginning its travel at a short distance from the sample but having acquired its rate when contact was made with the upper aspect of the sample. The machine sensors allowed measurement of the travel and compression stress throughout the procedure. An A/D card connected to a microcomputer was used to store data. Acquisition (8 points/s) was carried out using a special software specific to the card (Winview®). Data were then transferred to a spreadsheet (Excel, Microsoft®). For each sample, the test was interrupted once the first deflection of the stress/strain curve was obtained.

Deformation at the time of failure was measured, and elasticity modulus was calculated in the first, straight section of the strain deformation curve.

2.7. Statistical analysis

Ultrasound wave conduction rate: A comparison was performed on the conduction rate in the groups before any treatment.

After processing we compared the conduction rate before and after processing for each sample. The difference determined the delta-VC, which was the parameter studied for statistical tests.

The parameters studied were stress to failure, deformation at the time of failure, and elasticity modulus.

A non-parametric analysis of variance was performed (two factors: Groups of 4 samples, treatment) for each factor studied (Friedmann's test). When the test was positive, match comparisons were realized between each treatment studied and Newmann–Keuls test was used.

The α risk was defined at 5% level.

3. Results

3.1. Data concerning the delipidation group was lost as a consequence of a handling mistake

3.2. Results of measurements performed:

The mean results (±SD) are resumed in Table 1.

Before treatment, conduction rate at the time of inclusion was not significantly different between the four groups ($p = 0.32$). After treatment analysis of variance was significant for each parameter.

For the Delta VC parameter, the Newmann–Keuls test found a significant difference between the control group and the treated groups (Fig. 2). The results of the 6 M urea treated group were significantly different from the other results obtained with the other treatment and from the control group. No significant difference was

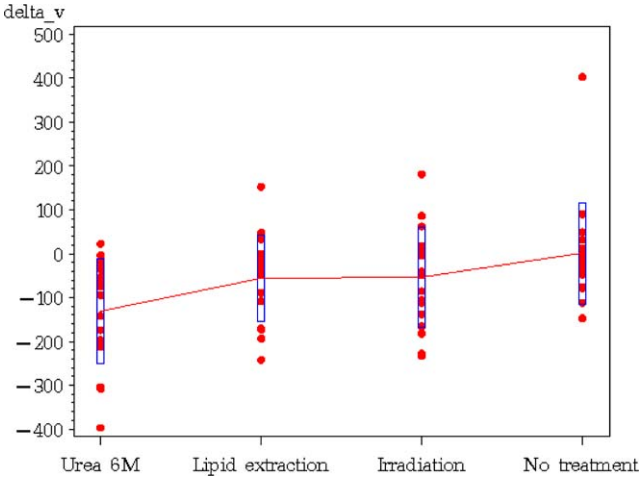

Fig. 2. Delta V before and after treatments.

Table 1
Mean results of the tests performed

Group	No treat.	Urea 6 M	Lipid extr.	Irradiation	p
V before TT (±SD)	2245±150	2232±152	2264±147	2229±150	0.32
V after TT (±SD)	2246±115	2100±173	2209±143	2175±125	
Delta VC (±SD)	0.4±115.4	−131.6±118.8	−55.7±98.5	−53.6±113.4	$<10^{-4}$
Sigma failure (±SD)	13.5±3.1	8.9±3.9	12.2±4.1	12.3±4.3	4.3×10^{-3}
Eps failure (±SD)	3.1±1.0	2.5±0.6	3.2±0.8	2.7±0.9	$<10^{-4}$
E (±SD)	876.8±331.6	646.1±359.0	761.9±268.0	817.2±282.5	3.3×10^{-2}

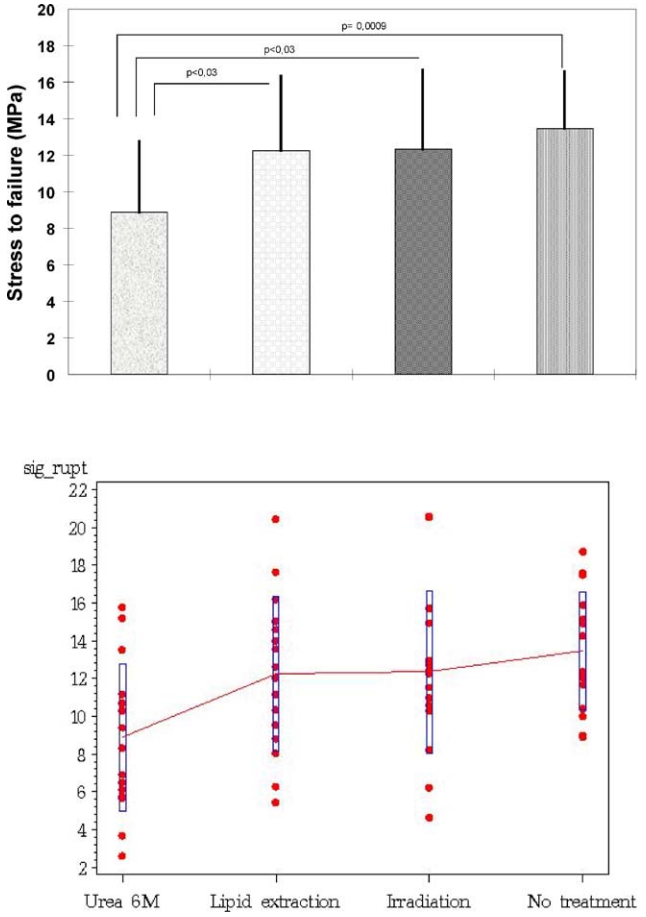

Fig. 3. Mean results of the stress to failure in the different treated groups.

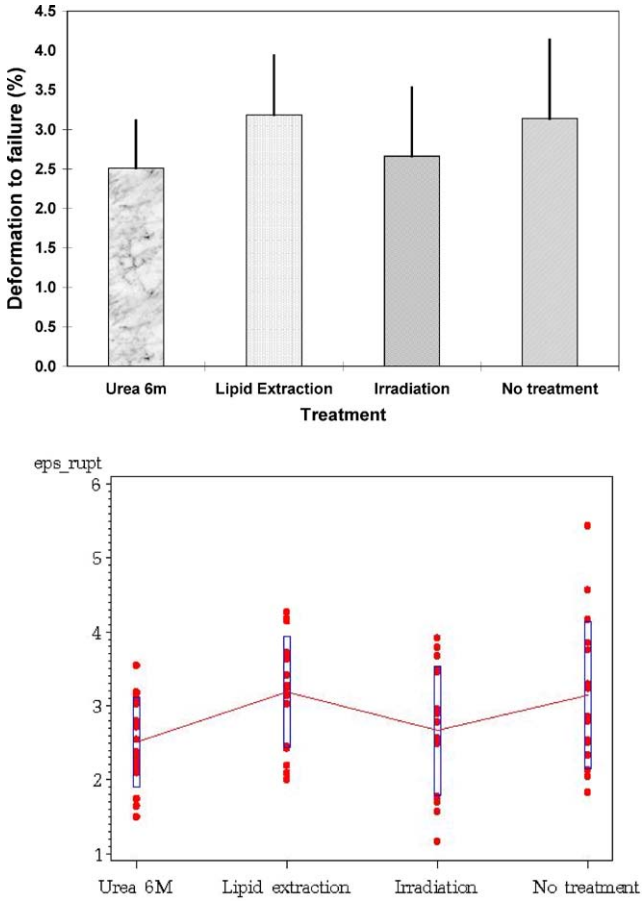

Fig. 4. Mean results of the deformation to failure in the different treated groups.

found between the group treated by lipid extraction and the irradiated group, but results were significantly different in one part from those obtained with the 6 M urea treated group, and from those obtained with the control group in a second part. For the measurement of deformation at the time of failure and stress to failure (Figs. 3 and 4), 6 M urea was significantly different from the others. No significant difference was observed between irradiation, lipid extraction and the control group.

4. Discussion

4.1. Measurements of rate of travel of the ultrasound wave

Abendstein et al. [14] found a constant relationship between the modulus of elasticity during compression determined by ultrasounds and the modulus of elasticity measured mechanically. Ideally, this formula can only be applied if the acoustic length is clearly greater than the mean diameter of pores and the dimensions of the surface area of a specimen value [15,16]. The value of the stress to failure is also closely related to the propagation rate measured [14]. Samples tested by these methods should be of adequate length. Ashman [15] proposed cubes of 12 mm on each side and advised against the use of samples not allowing a propagation of at least 5 mm. The transmission frequency used in our study, i.e., 2.25 MHz, corresponds to a 1-mm wavelength, greater than the mean diameter of pores in human trabecular bone (0.1–0.5 mm). This value is similar to those used in other published series, but it cannot claim to attain Young's modulus, considering the dimensions of the sample that are greater than the acoustic wave length.

4.2. Test conditions

Samples were tested after complete gradual thawing at room temperature. They remained moist throughout all handling procedures. Conduct of all tests in a continuous session before processing and then in a continuous session after processing, for ultrasound wave and for mechanical tests, allowed us to assume that conditions for measurement remained constant. The

lack of difference, regarding the ultrasound wave travel rates within the samples collected from a given femoral head, seemed to confirm that the choices made for preparation of the samples were valid. The choice of samples obtained from the central part of each femoral head (oriented in accordance with their situation in vivo), of small size, allowed the obtention of samples with identical characteristics, which would not be possible with samples of greater size. Thus mechanical test comparisons could be conducted, with the control sample, for each group of samples and liberation from inter-subject differences could be ensured [10,12,17].

4.3. Mechanical tests

Because of the speed used in this study, which was less than 10 m/s, the effect of the fluid phase, on the results obtained, need not be taken in account [18,19]. Stress direction on the studied samples, being orientated in accordance with its initial physiological situation, was consequently identical for all the samples tested [12,20,21]. Even though care was taken to grease the machine plates, the stresses of friction on the interface cannot be neglected—a reminder of the limits of any mechanical compression test. However, the results obtained were of the same order of magnitude as those reported in the literature [13,18,20], and the aim of the study was not to characterize trabecular bone as such, but rather to evaluate the effect of different sterilization processing methods on the mechanical properties of processed trabecular bone tested under similar conditions.

The protocol of rehydration and conservation between tests in physiological saline is questionable, but constitutes the usual method applied in surgery, and the protocol was identical for all the treatments tested, particularly for delipidation and delipidation together with 6 M urea treatment. The significant differences in the results of mechanical tests, observed between these two methods, cannot be attributed to the rehydration protocol.

4.4. Discussion of the results

A high consistency in the results obtained was observed between the measurement of travel time of the ultrasound wave and those carried out in the setting of mechanical tests: stress to failure and deformation to failure. This consistency of results is in agreement with the data in the literature [15,18,19].

Gamma irradiation at the doses used only slightly altered the measurements performed on the bone, which underwent processing. This result is consistent with the rare data in the literature [4,8]: the latter do not mention any significant effect of gamma irradiation at the doses used in orthopedic practice. In our context, the deterioration in the ultrasound wave conduction rate was poor (2.4%), but significant. The results obtained tends to demonstrate an effect of irradiation on trabecular bone tissue, which is also detected by conventional mechanical tests and which was not previously reported.

Lipid extraction produced a significant decrease in the acoustic conduction rate (2.5%); this variation was low and was also measured by mechanical tests.

Processing with 6 M urea significantly deteriorated the measurements performed on the bone which underwent processing; the fall in the travel rate was nearly 6% and the mean stress to failure showed important (34%) and significant differences between the different groups.

Comparative results obtained with the two methods used are consistent with a low structural deterioration when using irradiation, slightly lower (and significant) for lipid extraction, and high when processing with 6 M urea. These hypotheses are compatible with the action mechanism of the different processing methods used: physical processing for irradiation, which, at the doses used, does not greatly alter the protein structure of the material exposed. Lipid extraction, thus weakly activates denaturing agents. Urea acts by deteriorating the tertiary structure of proteins. At a 2 M concentration, it is already able to produce a major deterioration in globular proteins [22]. Fibrillar proteins, such as type I collagen, which is more resistant to the denaturing process, can be deteriorated at concentrations as high as those used (6 M). This is in agreement with the amplification observed for variations in the value of the stress to failure, suggesting a processing-related architectural deterioration, in relation to denaturing of the collagen framework, which is not observed with conventional treatments [4,8,23].

A high level of safety, including the process against "unconventional" protein agents such as the prion, necessarily alters the framework structure of treated bone and reduces its mechanical properties.

At present, the trabecular allograft is most frequently used in orthopedic surgery for acetabular reconstruction in reinforcement of prosthetic total hip replacement. The mean stresses tolerated by a hip are approximately 1–3 MPa in the acetabulum [12,18,24,25], with peak stresses which can reach up to 8.8 MPa [12]. The success of reconstruction depends on the resistance of the material employed to complete graft osteointegration: this must occur within a sufficiently rapid time period, i.e., less than the limits of resistance to fatigue of the reinforcement material used. From a mechanical standpoint, use of the reinforcement acetabular device seems to be very important, particularly when treated bone is used for the bone reconstruction.

From the standpoint of the tissue bank, such processes will not be applied to produce solid pieces when grafts are provided by old donors with

osteoporosis [26], for example in the case of fracture of the femoral neck.

5. Conclusion

Various deteriorations associated with processing were following investigation of the mechanical properties of trabecular bone from human femoral heads and evaluation of the effect of different sterilization processing methods. Measurement of the ultrasound wave propagation rate in the samples revealed a deterioration of 2.4% in the rate measured, compared to controls, resulting from gamma irradiation at doses of 30 kGy, which was significant. It revealed deterioration of 2.5% in the rate measured, which was significant, for lipid extraction with acetone and alcohol. It revealed 5.9% deterioration in the rate measured for complete processing with urea 6 M, which was highly significant. These deteriorations resulted in the same effects when measurement of the stress to failure was performed with conventional mechanical compression tests. Although these effects were significant, but low, for irradiation and lipid extraction in the samples observed, they were however clearly significant for urea 6 M, corresponding to a loss of 30% in values measured in the control sample for the stress to failure. In orthopedic surgery, care must be taken when using some treated bone graft with protocol including Urea 6 M, with systematic use of reinforcement device or osteosynthesis. High consistency in the results obtained between the measurement of travel time of the ultrasound wave and measurement of stress to failure incite us to use protocol with ultrasound as a routine quality test which can be performed before furnishing bone graft to the surgeon. Maybe this test will allow us to extend indications of bone procurement in multi-organ collection, particularly in older donors.

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