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The processing and characterization of animal-derived bone to yield materials with biomedical applications. Part III: material and mechanical properties of fresh and processed bovine cancellous bone

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Conversion of bovine cancellous bone to a useful biomedical xenograft material involves several processing steps which include boiling, defatting and deproteination (i.e. bleaching). This study has shown how these processes can influence cancellous bone modulus and strength. It was found that prolonged boiling in water for six hours followed by NaOCl bleaching had a deleterious effect on the overall strength of the bovine bone. In contrast, bone samples subjected to only moderate boiling (1.5 hours) exhibited a 22% stiffness increase due mainly to the effects of drying. The same stiffened samples, when subjected to the bleaching procedure, retained some strength with only a small reduction in moduli values. It can be concluded that careful control of defatting and bleaching procedures on bovine bone is able to give a strong, albeit, brittle material with preservation of the original bone architecture. The bone xenograft materials are worthy of further investigation in *in vivo* clinical trials to assess their performance in contact with biological fluids.

1. Introduction

Abattoir-derived animal bone waste constitutes a potentially useful source of materials suitable for biomedical purposes. New Zealand meat industry bone material has been traditionally used in the production of fertilizer and bone meal. In previous papers [1, 2], we have described the processing and characterization of animal derived bone in order to produce value-added materials. This has involved the production of defatted/deproteinated bovine condyle cancellous bone cubes as well as powders generated directly from milling of defatted/solvent cleaned/boiled/bleached bone or from acid-digestion/sodium hydroxide (NaOH) reprecipitation of bone materials. The bone cubes can be potentially used as bone xenografts or cavity fillers [1] whilst the milled and acid-digested/reprecipitated bone powders have shown promise in experiments [2] where they have been trialled as a drug delivery agent, enzyme immobilization substrate and as a feedstock powder for plasma-spraying on titanium metal substrates.

An important aim of the previously described work was to prepare an implant material which had the property of being cuttable by surgeons as well as having an aesthetically pleasing appearance even after cutting or shaping. To meet these requirements, defatting and deproteination procedures had to be tailored to produce an implant material that was cuttable and bleached to the core. In the present study we report elastic modulus and yield stress data gathered on processed bovine cancellous bone so that its potential as a shapeable filler material in structural bone can be assessed. This study has determined how these mechanical properties are affected by the two principal processing stages necessary for obtaining potential orthopaedic implant materials: defatting (removal of marrow and fat) and bleaching (which involves significant removal of collagen).

The bone being tested in the specimens is derived from the interior of the bovine femoral condyle and hence consists of cancellous bone. The nanometer scale structure of cancellous bone is similar to the structure of the dense cortical bone of the femoral shaft. Apatite crystallites, approximately 40 nm in length are placed lengthways along and within the collagen fibrils. These apatite-impregnated fibrils blend together into millimeter

length fibers. As revealed in our previous studies, removal of most of this organic matrix still results in a largely intact, albeit weakened structure. This shows that despite the intimate incorporation of the collagen into the bone matrix, the mineral portion of the bone matrix is largely continuous in nature [3]. There is evidence that the material composition of cancellous bone tissue is slightly different to that of cortical bone tissue, this being based on measurements of ash weight. Gong *et al*. [4] found that ash weight of cortical bone tissue was consistently higher than that for cancellous bone in four animals species tested, including humans. The ratio of ash to organic material in cancellous bone was found to be 2.18 compared with 2.48 for cortical bone. This lower state of mineralization was thought to be related to a higher rate of turnover in cancellous bone.

The mechanical behavior of cancellous bone is strongly affected by its millimeter scale architecture. Cancellous bone assumes a range of forms from a delicate three-dimensional array of straight and curved rods to a dense network of plates whose architecture is dependent on position in the bone [5]. The dimensions of these plate-like or rod-like structural elements and the way that they are connected have a profound effect on mechanical properties [6]. Cancellous bone is generally stiffest in principal directions of plate alignment. Thus cancellous architecture at the end of the long bones is characterized by cancellous plates which are aligned in principal directions of loading.

In this study, modulus and strength measurements were obtained by compression testing of samples cut in such a way that the loading axis was generally coincident with one of the bone's principal stiffness directions. This also helped to minimize shear coupling during measurement. Thus, a preliminary stereology study was required to determine the principal bone plate directions for cutting the samples for measurement of mass, modulus and strength afterward.

2. Materials and methods

The distal end of a fresh, frozen bovine left femur was sectioned using a bandsaw to expose cancellous bone surfaces: a flat anterior facing surface was exposed (Fig. 1b) and one centimeter thicknesses were removed from lateral and medial condyles (Fig. 1a) to expose lateral and medial facing surfaces. The surface marrow was removed with high pressure air, the exposed bone sanded, brush painted with black paint to highlight surface features and photographed. High contrast images (Fig. 2) were produced from photographs of the exposed bone surfaces. The angular orientation of bone plates was estimated by determining the mean intercept length as a function of angle. A grid of parallel lines was laid across each photo-image and the number of bone/marrow interface intercepts was recorded. This procedure was repeated for nine different orientations of the lines to the bone (20° apart). The inverse of the number of boundary intercepts (i.e. the mean intercept length, MIL) was plotted on radar diagrams (see Fig. 1a and 1b).

Six test samples (Group 1) were prepared for a pilot study. Slabs of bone, 8 mm thick were cut by bandsaw from the femoral condyles. Further cuts to individual slabs were made using a miniature modeller's saw (blade thickness 0.25 mm and diameter 30 mm) to produce bone samples of approximate dimension 24 mm × 8 mm ×

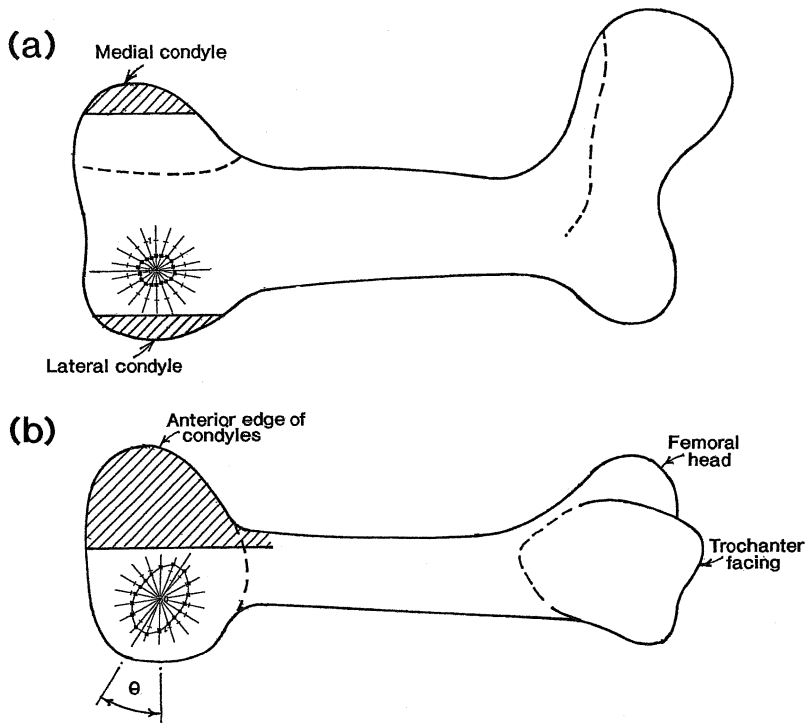

Figure 1 (a) A 1 cm thickness was cut from the lateral edge and the medial edge of the condyles. The MIL diagram [insert-lateral condyle] was obtained from exposed cancellous bone after the anterior surface was removed as in Fig. 1(b). Samples were cut parallel to this direction. (b) A 3 cm thickness bone slice was removed from the anterior surface of the condyles. The MIL diagram (inset) was obtained from exposed cancellous bone from the lateral condyle (refer to Fig. 1(a)). Samples were cut parallel to this direction.

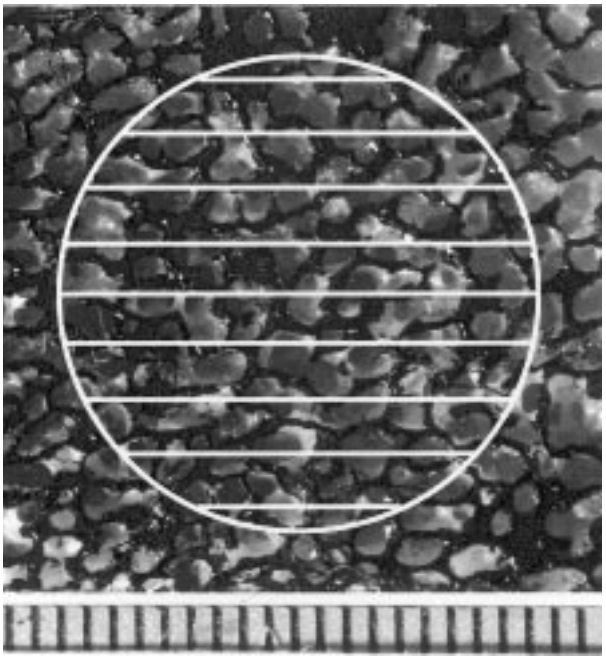

Figure 2 An example of a high contrast image of the cut bovine bone (lateral condyle front view). The scale at the bottom is in divisions of 0.5 mm. The circle with white lines represents the grid used for marrow/bone boundary crossing statistics. Lines in this picture are parallel to the supero-inferior direction.

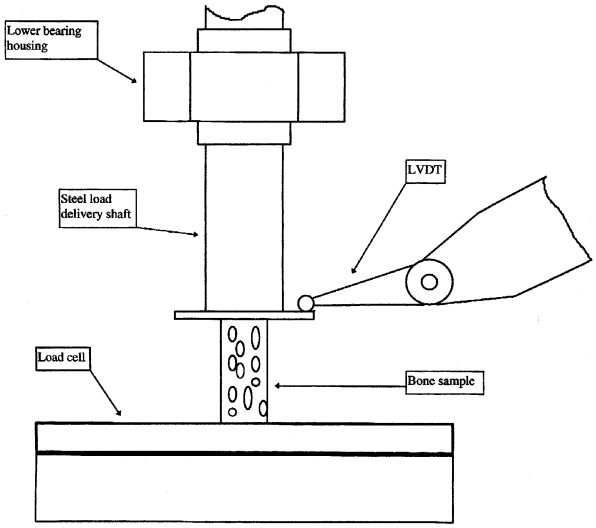

Figure 3 Close-up schematic of load-deflection measurement system.

8 mm. The long sides were cut in most cases parallel to the directions indicated in Fig. 1a and b (i.e. parallel to the long axes of the MIL ellipses). Test pieces were fine sanded to 24 mm × 6 mm × 6 mm in a special jig which supported them under light pressure so that their opposite faces remained parallel to each other. Samples were then held in a mill and cut to 20 mm length with parallel ends to produce samples of final (average) dimension 20 mm × 6 mm × 6 mm.

Nineteen samples (Group 2) were cut from the distal condyles of a second fresh, frozen bovine femur at the same angles as those of the Group 1 samples, but with some changes to the preparation protocol: the sanding process was omitted as it was found that sanding generally resulted in grit particles becoming entrapped within the surface cavities of the cancellous bone. Instead, Group 2 samples were cut on a modeler's saw to approximately 6 mm by 6 mm in cross-section and then cut on the mill to 20 mm length.

Specimen mass was measured on fresh bone and after the defatting and bleaching/deproteination processing stages by weighing the samples on a miniature balance (Mettler Instruments AG, Model AE 200-S). Stiffness measurements were obtained using a purpose built compression testing system depicted schematically in Fig. 3.

The test specimen was placed atop a load cell (9257A; Kistler Instruments AG) and compressed by a steadily increasing force delivered through a steel shaft held between two low friction bearings. Displacement between the end of the shaft and the face of the load cell was measured by a linear variable differential transformer (LVDT), accurate for submicron displacement measurements.

Load cell and LVDT calibration data were checked separately by placing known masses on the load cell and using slip gauges of known thickness for the LVDT. The whole system was tested by obtaining load/deflection data from a material of known compressive modulus (aluminum 6063 [7]). In a final test, a 4 mm diameter, 25 mm long cylinder of this reference material was placed longitudinally in the test machine and subjected to a load up to 750 N. The slope of the linear portion of the load/deflection curve gave a stiffness of 33.7 N/mm. The calculated modulus was 67 GPa. The literature value for the compressive modulus for the reference material is 69.7 GPa which places the measured modulus within 4% of the published compressive modulus for aluminum 6063 [7].

The bone specimens were cyclically loaded in the direction of their long axis until the response was stable as recommended by Linde and Hvid [8] and then loaded at a rate of 3.5 to 6 N/s to a maximum strain of about 0.6%. The displacement output of the LVDT was displayed on a meter and the loading was stopped when the maximum strain level was reached. Continuous data were collected for loading and unloading. Digitized data were placed in a text file and this was copied to a spreadsheet. A regression analysis on the (load increasing) load deflection curve was performed between 0.1% and 0.4% strain. Each stiffness K, was taken as the regression line slope. An apparent modulus E, was calculated from the stiffness data using the following equation:

$$E = \frac{Kl}{A} \tag{1}$$

where l = sample length and A = sample cross-sectional area.

Several samples were chosen at random and taken to yield at the end of each of the two intermediate process steps with all remaining samples being tested for yield strength at the end of the last processing stage. Yield strength was defined from the intersection of the load/deflection curve with a line parallel to the line defining the elastic modulus but offset by 0.1% strain.

The defatting and bleaching processes used for generating the bone samples in Group 1 and Group 2 followed the standard methods developed and discussed in an earlier report [1] but with some modifications to allow separation of individual samples for analytical and measurement purposes.

In general, the following conditions were used:

1. Samples were pressure cooked in a domestic stainless steel 15 psi pressure cooker. During this processing step, samples were kept separate by using labeled individual 150 ml glass flasks in the cooker. Aluminum foil was used to cover the flask tops.
2. The solvent reflux step (utilizing BDH Anala-R methyl acetate) was performed in standard quickfit reflux apparatus but with each individual bone sample placed in a separate quickfit set so that samples could be identified.
3. The bleaching/deproteination step was carried out in separate flasks using a volume of 100 ml of bleaching solution (NaOCl) to allow identification of individual samples. A holed stopper was employed for degassing purposes. Soaking with ultrasonication as recommended in Johnson *et al*. [1] for optimum bleaching/deproteination was not used for the bone samples destined for mass, modulus and yield strength measurements as it was believed this would lead to some destruction (by chipping off of edges or premature breakage) of the materials and so render them unusable for measurements (the sizes of samples subjected to the mechanical measurements were, by necessity of the measurement technique used, smaller in physical dimension than the typical cubes processed as described in Johnson *et al*. [1], which are more robust to ultrasonication treatments). The disadvantage of not using ultrasonication was counteracted by the fact that the cubes (being of smaller dimensions) were more easily penetrated by the bleaching solution than cubes of larger dimensions.

Specific conditions used for the Group 1 set of (6) bone samples were thus:

a. Pressure cooking in 100 ml of distilled water for 6 h with a water change after 2 and 4 h of cooking;
b. Solvent refluxing in 50 ml of methyl acetate for 1 h;
c. Soaking for three days in l00 ml of 1% NaOCI solution with a solution change daily.

Specific conditions used for the Group 2 set of (19) bone samples were thus:

a. Pressure cooking in 100 ml of distilled water for 1.5 h;
b. Solvent refluxing in 50 ml of methyl acetate for 1 h;
c. Soaking for 20 h in 100 ml of 1% NaOCI solution.

Measurements were made on fresh thawed samples before the first (a) process step, after the defatting step, (a) and (b), and finally after the bleaching (c) step. For the Group 2 set of samples, the defatting processing step was less vigorous relative to that used for samples in Group 1 (1.5 h of pressure cooking as opposed to 6 h for Group 1).

3. Results

The defatting process reduced measured moduli for each Group 1 sample to, on average, 72% (s.d. 5%) of the untreated (fresh) value[1]; for Group 2 the opposite occurred, individual moduli[1] were on average increased to 115% (s.d. 26%) of the untreated value. Despite their higher initial density (which was indicative of a greater bone density), the samples of Group 1 lost, on average, proportionately more of their mass after defatting they were 39% (s.d. 1%) of their original mass; compared with 43% (s.d. 6%) for Group 2. Average strain at yield (0.1% offset) after defatting was 0.7% (s.d. 0.2%) for Group 1 and 0.9% (s.d. 0.3%) for Group 2.

Bleaching the Group I samples resulted in a chalk-like product that was too brittle and weak to test. In contrast, the Group 2 samples could be handled and tested mechanically after the bleaching stage. Bleached Group 2 moduli[1] were found to be on average 83% (s.d. 18%) of their untreated value. In all but two instances (samples 5 and 10, see Table I), moduli were reduced. Bleaching also resulted in the further loss of material from samples: bleached Group 2 samples were on average[1] 86% (s.d. 3%) of their defatted mass. The average (0.1% offset) yield stress of the end-product (Table I) was 4.1 MPa (s.d. 2.3 MPa). Average strain at yield was 0.7% (s.d. 0.2%) for the Group 2 samples.

4. Discussion

Elastic moduli and yield stress data measured on the untreated bone were similar in value to bovine cancellous data collected from several other studies (see Table II). Average moduli were lower for Group 2 compared with Group 1 and this may be due to a lower volume fraction of bone in these specimens as indicated by their lower material density. To avoid damage at intermediate processing steps, measurement strains were less than 0.6% unless samples were intentionally compressed to their point of yield. This value of strain is much less than the yield strain for bovine distal cancellous bone reported by Turner [9], which is *ca*. 0.9%, and the range of yield strains (1.0%–1.2%) reported in the present study (see Table I). At the defatting stage the average yield strain over the four samples tested (Group 1 and 2) was 0.8%, suggesting that the 0.6% criterion was also suitable for avoiding significant damage on tested bone.

The results of the stereological analysis prompted us to test samples in a direction which was predominantly antero-posterior (see Fig. 1). The evidence for this direction being a principal one is supported by Turner's data [9]; given that bone samples tested in that study in the antero-posterior direction had an average modulus that was 38% and 160% greater than samples tested in longitudinal (e.g. supero-inferior) and medio-lateral directions respectively (see Table II). Testing in a principal direction would minimize shear coupling (e.g. shearing of the sample during compression) and in this way improve the quality of the measurement. Measured moduli would also be at or near maximum and thus useful for comparison with other studies. Testing in an arbitrary direction can result in significantly lower modulus measurements. Errors in modulus associated with misalignment from the principal direction were

TABLE I Mass, density, modulus, yield stress (0. 1% offset) and yield strain data for fresh, defatted and bleached bone samples. Several samples did not complete the test program. Missing data is indicated by the '?' symbol. Bleached Group 1 samples 3 and 4 were too brittle and weak to test. Fresh Group 2 sample 1 was strained beyond the 0.6% test limitation. Bleached Group 2 samples 3 and 19 were damaged during processing

	Fresh					Defatted					Bleached				
	Mass g	Density $gmm^{-3} \times 10^6$	Modulus Nmm^{-2}	Stress Nmm^{-2}	Strain %	Mass g	Density $gmm^{-3} \times 10^6$	Modulus Nmm^{-2}	Stress Nmm^{-2}	Strain %	Mass g	Density $gmm^{-3} \times 10^6$	Modulus Nmm^{-2}	Stress Nmm^{-2}	Strain %
Group 1															
1	0.92	1,253.3	910.5	—	—	0.36	482.56	677.1	2.7	0.5	—	—	—	—	—
2	1.04	1,499.1	1,354.0	15.3	1.2	—	—	—	—	—	—	—	—	—	—
3	0.98	1,344.3	1,372.7	—	—	0.37	506.83	937.7	—	—	Damaged	?	?	?	?
4	0.99	1,366.8	1,300.4	—	—	0.38	530.68	1,020.6	—	—	Damaged	?	?	?	?
5	1.03	1,419.3	1,506.4	—	—	0.41	570.76	1,003.3	7.6	0.8	—	—	—	—	—
6	0.96	1,380.3	1,668.4	16.1	1.1	—	—	—	—	—	—	—	—	—	—
Average	0.99	1,377.2	1,352.1	15.7	1.2	0.38	522.7	909.7	5.1	0.7	—	—	—	—	—
Stan. dev.	0.04	81.5	253.7	0.6	0.1	0.02	37.6	159.1	3.5	0.2	—	—	—	—	—
Group 2															
1	0.79	1,171.4	325.5	—	—	Damaged	?	?	?	?	?	?	?	?	?
2	0.88	1,107.3	553.3	—	—	0.28	346.43	510.0	3.0	0.7	—	—	—	—	—
3	0.86	1,125.0	851.6	—	—	0.33	430.99	868.2	—	—	Damaged	?	?	?	?
4	0.83	1,127.0	608.7	—	—	0.27	374.32	51.9	—	—	0.22	296.4	378.1	1.4	0.4
5	0.94	1,207.1	352.8	—	—	0.35	451.87	502.3	—	—	0.29	367.4	400.9	1.9	0.6
6	0.86	1,177.6	572.1	5.6	1.1	—	—	—	—	—	—	—	—	—	—
7	0.90	1,234.9	809.1	—	—	0.42	578.93	835.5	—	—	0.36	499.2	601.0	—	—
8	0.84	1,220.0	755.7	—	—	0.35	517.24	936.9	—	—	0.29	425.2	703.1	2.9	0.5
9	1.02	1,318.8	869.0	—	—	0.51	661.37	831.4	—	—	0.45	575.9	546.4	3.6	0.8
10	0.99	1,252.2	730.2	—	—	0.48	605.95	1,095.0	—	—	0.42	525.3	772.5	5.7	0.9
11	0.93	1,228.2	1,086.7	—	—	0.40	522.21	1,123.7	—	—	0.35	457.4	1,010.6	6.1	0.7
12	0.95	1,257.3	1,430.5	—	—	0.47	621.36	1,318.4	—	—	0.42	555.3	1,296.7	8.7	0.8
13	0.89	1,196.2	479.0	—	—	0.20	543.01	640.3	—	—	0.35	470.4	431.2	3.3	0.9
14	0.94	1,225.6	974.2	—	—	0.47	611.50	1,184.0	—	—	0.42	547.7	859.2	6.5	0.9
15	0.85	1,171.9	1,112.8	—	—	0.38	526.53	1,218.3	11.7	1.1	—	—	—	—	—
16	0.82	1,176.4	807.2	—	—	0.31	451.02	769.3	—	—	0.26	377.8	567.7	2.9	0.6
17	0.85	1,169.2	525.4	4.7	1.0	—	—	—	—	—	—	—	—	—	—
18	0.96	1,235.0	691.6	—	—	0.43	551.20	740.4	—	—	0.38	488.1	386.2	2.4	0.7
19	0.98	1,259.6	909.9	—	—	0.49	625.96	1,685.1	—	—	Damaged	?	?	?	?
Average	0.90	1,203.2	760.3	5.2	1.1	0.40	526.2	925.7	7.4	0.9	0.36	471.9	662.8	4.1	0.7
Stan.dev.	0.07	53.2	275.8	0.6	0.1	0.08	93.2	327.0	6.2	0.3	0.07	84.8	283.0	2.3	0.2

studied by Turner and Cowin [10]. They reported that modulus would be in error by 9.5% at a misalignment angle of 10° and that this error would increase as angle increased. Their graph of modulus error versus misalignment angle shows that for a misalignment of 20° the average error will be *ca*. 25%.

The defatting process altered the elastic modulus of the bone samples. Defatting reduced Group 1 moduli but had the opposite effect on Group 2 bone which became stiffer. Defatting included a period of pressure cooking and the cooking time for Group 1 was six times longer than Group 2. In both cases the bone was tested in the

TABLE II Comparison of material properties obtained on untreated bovine bone samples in the present study with bovine femoral cancellous bone properties from several other studies. Figures in parentheses refer to the standard deviations of the measurements reported in each study

Study	Bovine bone description	Material density ($kg\ m^{-3}$)	Elastic modulus data [test mode]	Yield strength $N\ mm^{-2}$ [offset value]
Keaveny *et al*. [13]	Proximal tibia	Not provided	2380 (777); [compression]	21.3 (8.05) [0.2% offset]
Turner [9]	Samples cut from distal femur in longitudinal (L), mediolateral (M) and anteroposterior (AP) directions	L : 2126 (94) ML : 2004 (99) AP : 2080 (81)	L : 1036 (531) ML : 550 (216) AP: : 1429 (459) [compression]	L: 6.975 (4.098) ML : 5.023 (1.971) AP : 13.38 (4.831) [0.03% offset]
Ashman and Rho [14]	Distal femur	1739 (74.2)	2110 (850) [ultrasound]	Not applicable
Present study	Distal femur Group 1 Group 2	 1377 982) 1203 (53)	 1352 (253) 760 (276) [compression]	15.7 (0.6) 5.2 (0.6) [0.1% offset]

dry state. A review by Evans [11] on the effects of drying on cortical bone, has indicated that elastic modulus along with tensile and compressive strength, and hardness all increase; increases in compression modulus of 18–24% can be expected. Townsend *et al.* [12] studied the effects of drying on the mechanical properties of individual cancellous bone trabeculae, measured an average stiffness increase of 24%. The average stiffness increase (22%) measured on the Group 2 samples after the defatting process was comparable with the results cited above [11, 12] suggesting that the stiffening was mainly attributable to material changes associated with drying.

In many instances, processing altered the shape of the load deflection curve. Load-deflection curves on untreated bone for strains between 0.1% and 0.4% were characterized by a slope that increased with strain, i.e. a concave upward curve. Upon bleaching, the same samples exhibited relatively flat or decreasing (concave downward) slopes over the same strain range (see Fig. 4).

The prolonged period of pressure cooking of the Group 1 samples followed by the long bleach deproteination stage had a deleterious effect on the strength of Group 1 bone, resulting in bone that was too brittle (almost chalk-like) to be tested for yield stress. Reducing both the defatting and the bleaching/deproteination times for the Group 2 samples has undoubtedly resulted in a stronger product that has maintained a modulus value similar to bone in the fresh state but with a considerably lower yield strength. Bleaching from earlier studies reported on processing of bovine cancellous bone xenografts [1] is known to lead to *ca.* 97.5% removal of the intimately incorporated collagen from the cancellous bone, albeit with preservation of the original bone architecture. It was obvious from the present study that excessive bleaching causes a steep fall in yield strength. Careful control of defatting and bleaching processes can result in a strong but brittle material. Due to its brittle nature, the utility of processed bovine cancellous bone as a xenograft material in areas subjected to mechanical loading may be limited. Further investigations involving *in vivo* clinical trials and mechanical measurements where the xenograft is wetted with biological fluids may be necessary in order to establish limitations on the applications of processed bovine cancellous bone as a biomedical implant replacement material for bone subjected to significant mechanical loading.

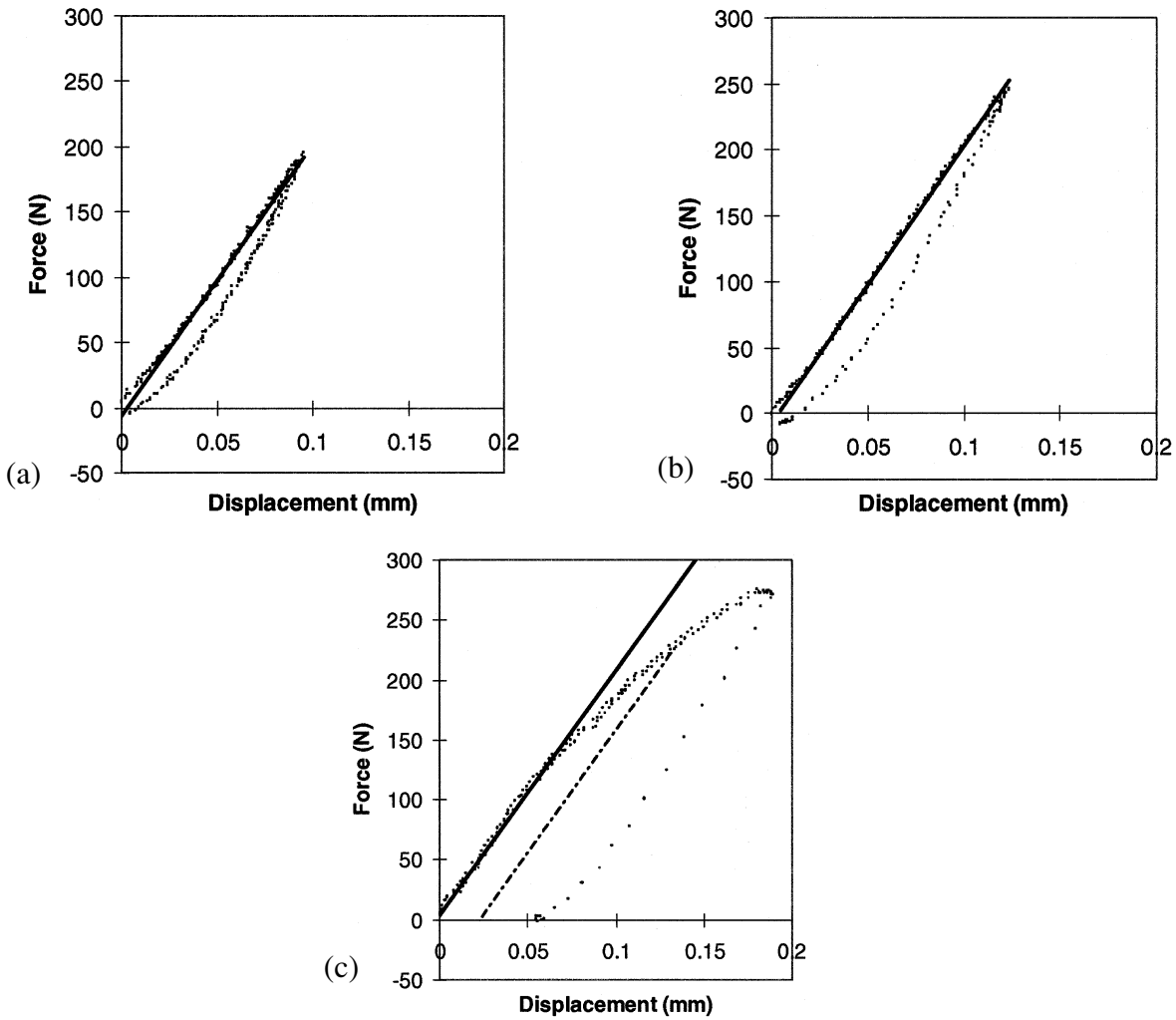

Figure 4 Load-deflection curves for sample 11 of the Group 2 sample set; (a) fresh, (b) defeated and (c) bleached bone. Regression lines were fitted to data points on the load increasing part of the cycle between 0.1% and 0.4% strain. Yield force for calculating yield stress (refer to (c) above) was defined by the intersection of the strain offset (dashed line) and the load/deflection curve. For 20 mm long samples the strain offset was 0.020 mm.

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Note

1. Note that these quoted mean % change values and their standard deviations are obtained on a paired comparison basis by averaging over the actual numbers of tested samples involved in each processing category before and after the processing step and therefore are not specifically written in Table I but can be computed using the data presented.

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