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Effects of hydrogen peroxide cleaning procedures on bone graft osteoinductivity and mechanical properties

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Abstract

Bone allografts are frequently used during orthopaedic trauma cases or other reconstructive procedures. Most allografts are processed and cleaned before use. Our goals were to determine if an improved cleaning procedure compromises the strength or osteoinductivity of a graft. We compared our improved cleaning procedure to our standard cleaning procedure on cortical bone allograft. The cleaning procedures are generally composed of a series of chemical steps with nonionic detergents, hydrogen peroxide, and alcohol under time and temperature control, subjected to ultrasonic agitation. We tested the compressive strength, impact strength, and shear strength following the standard and improved cleaning procedures. Osteoinductivity was tested in 4 groups, using the improved cleaning procedure with four different hydrogen peroxide cleaning times: 0, 1, 3, and 5 h. Osteoinductivity was evaluated *in vivo*, using a 28-day implant in the hamstring muscle of an athymic, nude mouse. Results demonstrated that osteoinductivity is maintained with cleaning in hydrogen peroxide for up to 1 h, and that compressive strength, impact strength, and shear strength were all unaffected by the improved cleaning procedure. The improved cleaning procedure therefore did not compromise the strength or osteoinductivity of cortical bone allografts in comparison to the standard procedure.

Introduction

Bone allografts are vital for skeletal deficiencies that occur from trauma, joint reconstruction, or other reconstructive procedures. There were over 986,000 bone grafts distributed in 2002 (American Association of Tissue Banks 2004), and their usage is growing.

Today, most cortical bone allografts are processed and cleaned before use (Mankin 1993; Boyce et al. 1999; Tomford and Mankin 1999). Allografts are typically cleaned physically and chemically to sterilize or help reduce the bioburden, and reduce the cellular antigens in the grafts. Chemical cleaning of grafts provides an additional level of safety over and above donor screening (AATB and FDA donor screening rules.), but it is important not to jeopardize the graft by cleaning it. Commonly used chemical methods employ aqueous solutions of detergents or surfactants, hydrogen peroxide or other peroxide, organic solvents, acids, and alcohol. Frequently, the chemical methods are used in combination with mechanical methods, such as pressure or vacuum, acoustic energy such as an ultrasonic bath, agitation, or centrifugation to facilitate the chemical cleaning and processing.

During processing, tissue is debrided, cut to specification, and cleaned. Debriding removes the

muscle, fat, and extraneous connective tissue from the recovered bone, either manually or mechanically assisted. Cut, debrided bone is cleaned using a standard procedure with a nonionic detergent soak in an ultrasonic bath followed by a static soak in denatured ethanol.

Cleaning grafts for safety can lead to associated problems in their mechanical properties, osteoconductivity, and osteoinductivity. Osteoinductivity and strength are critical properties of bone allografts. Osteoinductivity is the ability to form new bone by recruiting host pluripotent cells that differentiate, initially forming a cartilaginous template that becomes vascularized, ultimately forming mineralized bone (Wolfe et al. 1999). The strength of cortical bone allografts is important for load bearing applications, such as spinal surgery and joint reconstruction (Kummer et al. 1998; Head and Malinin 2000). Hydrogen peroxide (H_2O_2) is an oxidizing chemical with the potential to compromise osteoinductivity and bone structural proteins.

Sterilization methods are often used to process cortical bone in conjunction with cleaning or without cleaning, and are used for bioburden reduction, secondary sterilization, or terminal sterilization. New methods are constantly being sought that are more effective at sterilization and less destructive to the tissue. Most allograft bone that is processed outside the United States uses terminal sterilization with gamma radiation with exceptions such as Germany, Brazil, and Finland (Phillips and Morales 2002).

Sterilizing grafts for safety can also lead to associated problems in their mechanical properties, osteoconductivity, and osteoinductivity. Gamma radiation is known to reduce the mechanical strength of allograft bone and leave the bone more predisposed to fracture (Currey et al. 1997; Hamer et al. 1999; Akkus and Rimnac 2001; Mitchell et al. 2004) and has been shown to cause a decrease in osteoinductivity Munting et al. 1988; Ijiri et al. 1994). Temperatures above 60 °C can degrade the beneficial biological factors present in bone (Hallfeldt et al. 1995; Ito et al. 1995), while autoclaving will reduce bone's mechanical strength (Speirs et al. 1999). Lastly, ethylene oxide will destroy the osteoinductivity of demineralized bone (Munting et al. 1988; Aspenberg et al. 1990) and can impair new bone growth (Thoren and Aspenberg 1995).

We recently developed an improved cleaning procedure that cleans the grafts with sequential soaks in a nonionic detergent, hydrogen peroxide, and specially denatured alcohol (Recipe SDA 3-C, ethanol denatured with isopropanol) (U.S. Government 2002), with all soaks in a temperature controlled ultrasonic bath at 34 ± 1 °C. The objectives of this study were to therefore test the effects of the improved cleaning procedure on the osteoinductivity and mechanical properties of cortical bone, using the standard bone cleaning procedure as a control. The null hypothesis is that there is no difference between the improved procedure and the standard procedure.

Methods

Tissue preparation

Human cortical bone was recovered from donors with research consent according to American Association of Tissue Banks (AATB) guidelines (Woll and Kasprisin 2001). All tissue was stored at between -67 and -70 °C. Initially, the recovered tissue was thawed in an aqueous solution of Gentamicin (2 g /4 l H_2O) for greater than 30 min, debrided using a 6'' stainless steel wire wheel (Part #21575, Osborn International, Cleveland, OH) or scalpel, and cut and shaped using a band saw and/or milling machine (Sherline Products, Inc, Vista, CA). After this, the cortical bone samples were cleaned, either with a standard procedure or with the improved cleaning procedure.

Standard cleaning procedure

For the standard procedure, the cortical samples are cleaned by soaking in an aqueous solution of 1% Tween 80 (nonionic detergent) in an ultrasonic bath for greater than 30 min, then rinsed under running 35 °C water. This is followed by a static soak in denatured ethanol for greater than 60 min at room temperature, then rinsed under running 35 °C water.

The improved cleaning procedure

For the improved cleaning procedure, the cortical samples are placed into a 4.5 l stainless steel container with 4 l of a treatment solution. The container is placed into a temperature-controlled

ultrasonic water bath at 34 °C for all steps. The soaks consist of timed successive steps (Figure 1). The first chemical step is a 30-min soak in 0.1% Triton X-100. This is followed by three water soaks (5-, 5-, 10-min). The second chemical step is a 60-min soak in 3% hydrogen peroxide, used at a specific ratio of 14 cc per gram of bone. This is followed by three water soaks (5-, 5-, 30-min). The third chemical step is a 60-min soak in 70% alcohol soak (SDA-3C is used because a special

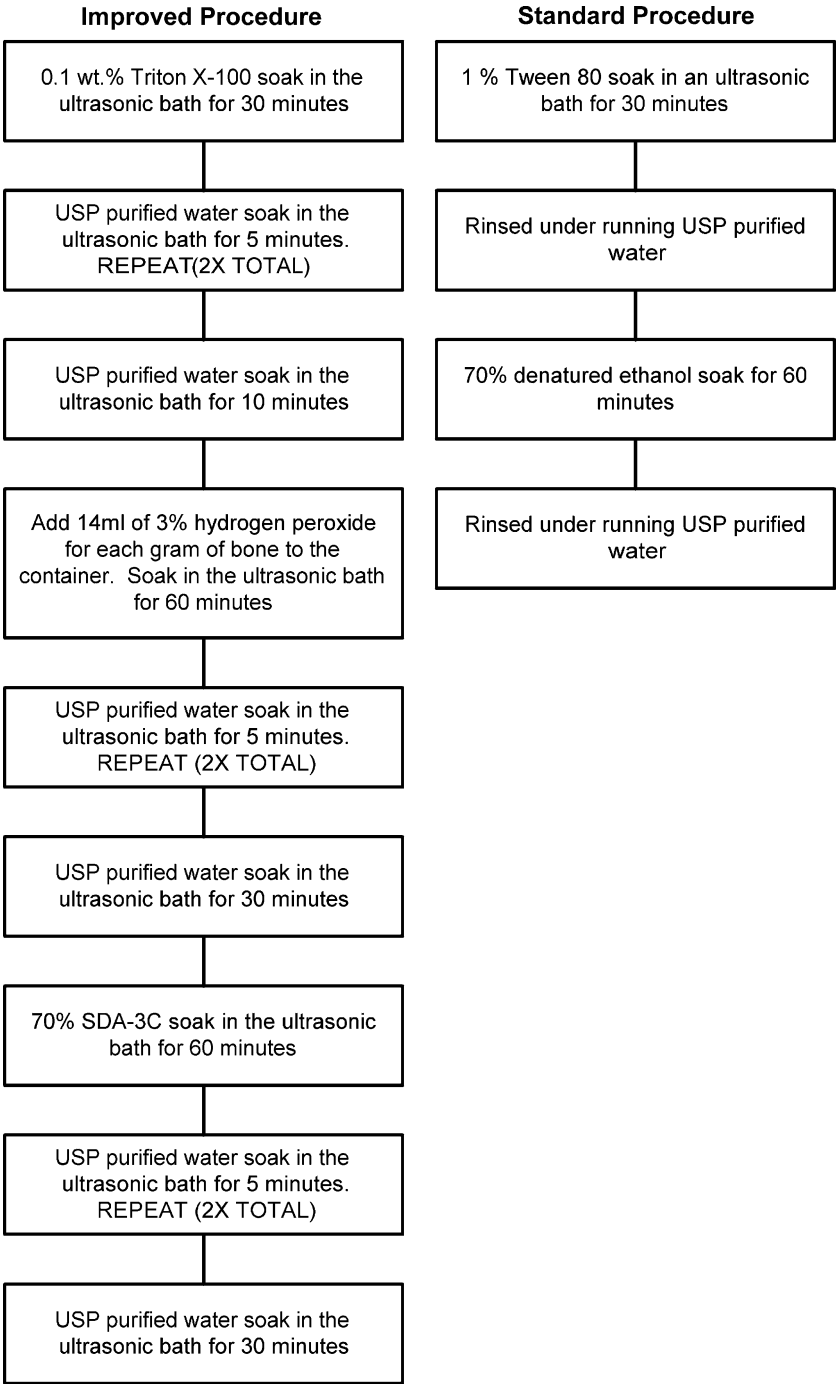

Figure 1. Flowchart of the standard and improved cleaning procedure.

government license is required to use pure ethanol). This is followed by three water soaks (5-, 5-, 30-min).

To assure the ultrasonic tank was operating within specifications, an ultrasonic cavitation meter (PPB-500, ppb, Inc., San Diego, CA) was used to verify that the energy flux of cavitation in the water bath was greater than 20 Watts per square inch (W/in^2). The meter measures cavitation of the imploding solution bubbles and also the sound waves produced.

In-vitro *tests*

Mechanical testing

As a primary goal, it was important to test the effects of hydrogen peroxide treatment on mechanical properties. Control samples were cleaned using the standard procedure. Test samples for compression testing and impact testing were cleaned with the improved cleaning procedure modified to use a 5-h H_2O_2 soak, to simulate exaggerated conditions. Shear testing was also performed.

Compression testing

Compression testing was performed because it is a simple material test and can make evident small changes in the mechanical properties of the bone matrix, and it is also the major loading pattern of bone *in vivo*. Compression cylinders (5.3mm × 5.3mm) were fabricated with orientations both longitudinally and transversely from the mid-diaphysis of human femora from donors 7 and 8 (age 39M & 61F). Donor selection represented both genders and different ages, as available. Femurs were chosen because they have the thickest cortical walls and this was necessary in the fabrication of the test cylinders. The samples were packaged as both frozen (-70 °C) and freeze-dried, to test the two methods currently used to preserve tissue forms. Longitudinal specimens were fashioned such that the loading axis of the bone corresponded with the loading axis of the specimen. Transverse specimens were fabricated such that the direction perpendicular to the loading axis of the bone (radial) corresponded with the loading axis of the specimen. Specimens for compression testing were divided into groups, separated according to cleaning treatment, donor, orientation, and storage. Additionally, the anatomical quadrant (medial, lateral, anterior, posterior) information was recorded for each specimen and the specimens were evenly divided among the groups. This is because the mechanical properties in a bone can vary within its anatomy and this eliminated this variation. The result is a total of 8 groups per cleaning process with 8 samples per group.

Freeze dried samples were rehydrated for at least 1-h prior to testing and frozen samples were soaked for at least 15-min prior to testing, both in normal saline at room temperature. Samples were loaded to failure in uniaxial compression, at room temperature, at a strain rate of 0.01 s^{-1} (Mow and Hayes 1991) using an Instron universal testing machine (Instron Corporation, Canton, Massachusetts; Model #4204). The force (load) and displacement data were electronically recorded for each sample, and maximum and yield stress were calculated. A diagram explaining the experimental design is presented in Figure 2a.

The compression study was designed using a prospective power analysis. A sample size of 8 was required to observe an effect size of 5% with an alpha-level of 0.05 and 90% statistical power (1-Beta) level. Data was analyzed using analysis of variance (ANOVA) with statistically significant differences at $p < 0.05$ (S-PLUS, Insightful Corp, Seattle, WA).

Impact testing

Impact testing was performed because it simulates a real world use of the bone under surgical conditions. Specimens for the impact testing were machined into 7-mm lordotic anterior cervical fusion (ACF) grafts from the mid-diaphysis of radii and ulnae from donors 9, 10, 11, and 12 (age 46M, 21M, 60M, & 62M), and were packaged as both frozen and freeze-dried. Radii and ulnae are used because of the cortical bone thickness and diameter. ACF's were chosen because they provided consistent sized cortical specimens with a reproducible shape, and are small [worst-case]. The posterior wall thickness was measured for each specimen and then the ACF's were ranked in order of increasing posterior wall thickness. Previous research (unpublished) showed that posterior wall thickness was associated with failure level. Specimens were then alternately assigned to the standard cleaning and then the improved cleaning group, in rank order. All the specimens in each

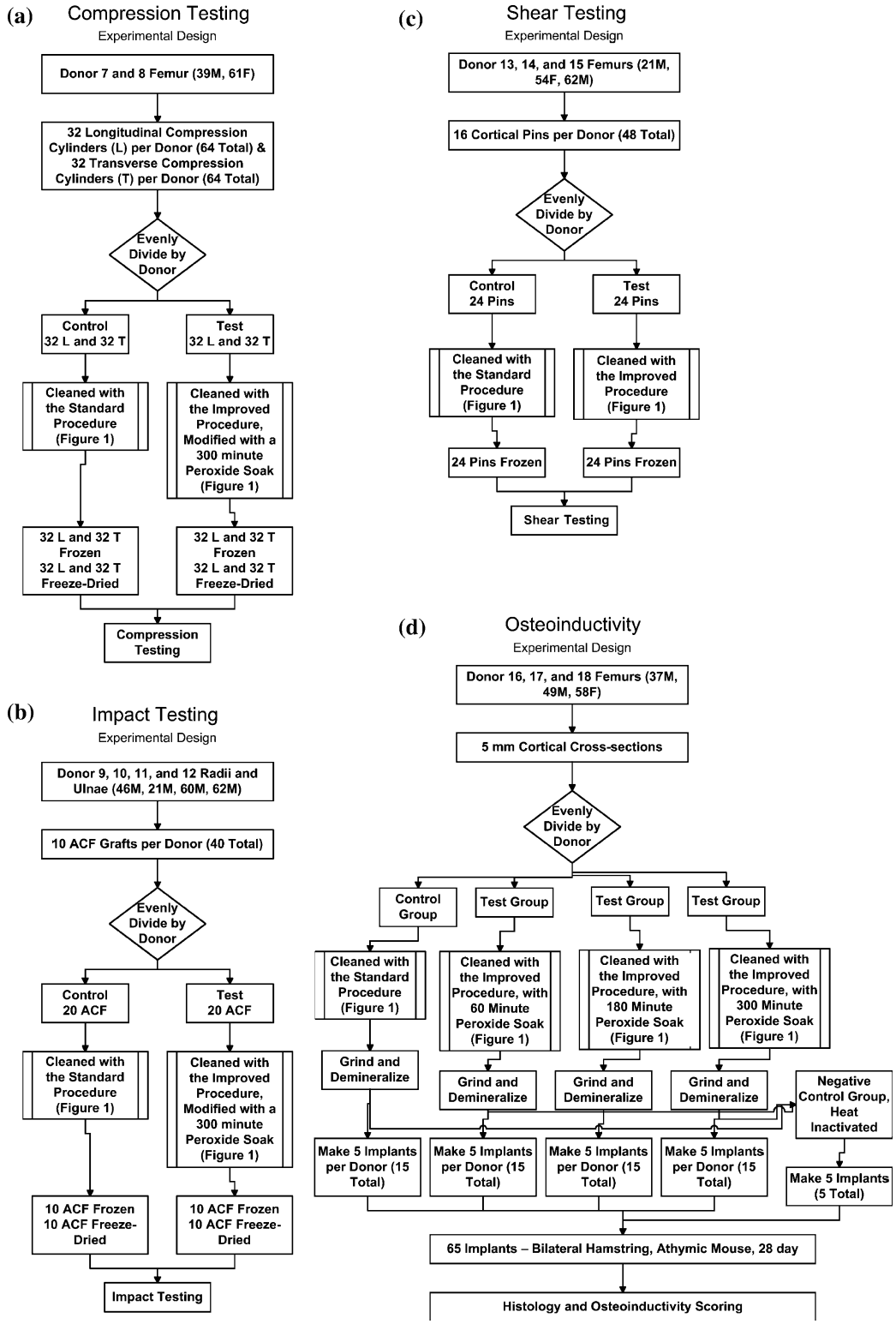

Figure 2. Experimental design for the (a) compression testing, (b) impact testing, (c) shear testing, and (d) osteoinductivity testing.

control and corresponding treatment group were from the same donor to minimize donor variability. The result is a total of 4 groups per cleaning process with 5 samples per group.

Each impact specimen was placed into a fixture (which included a 6° bevel), with the anterior side of the sample facing up. Samples were then secured into place using 3 Nm of torque (approx 65 N parallel to loading axis of ACF), and impacted starting at 5-cm with 1-cm increments until failure using a Synthes ACF impactor (Synthes Spine, Paoli, PA; Model# 396.398), simulating the orthopaedic use of an ACF. The specimens were not hydrated during testing. After each impact, the specimen was removed and examined by eye for failure. Failure was defined as a crack completely through the cortex. The total number of impacts and the height of each impact were recorded along with a description of the specimen after failure. For each specimen, the total kinetic energy absorbed was calculated. For each impact: $KE_{impact} = 1/2^{*}m^{*}V^{2}$, where m is the mass of carriage (840 g), V the carriage velocity = $(2\ gh)^{0.5}$, g the gravity acceleration, h the height. Total: $KE_{Total} = \sum_{1}^{n} KE_{impact}$. A diagram explaining the experimental design is presented in Figure 2b.

A prospective power analysis was performed to determine the sample size necessary for the impact study, to observe an effect size of 5% with an alpha-level of 0.05 and 80% statistical power (1-Beta) level. However, the sample size of $N = 1605$ is not possible from one donor, and a study with $N = 5$ specimens per group was conducted due to tissue availability and to look for gross differences. Data was analyzed using analysis of variance (ANOVA) with statistically significant differences at $p < 0.05$.

Shear testing

Shear testing was performed because it is a simple material test and can make evident small changes in the mechanical properties of the bone matrix, and bone is an anisotropic material and is weakest under shear load. Torsion testing was not conducted because bone fails in a shear mode under torsion and we felt the shear testing was sufficient. Shear specimens were prepared from the mid-diaphysis of femora from donors 13, 14, and 15: 21-year-old male, 54-year-old female, and 62-year-old male ($N = 8$ for all groups). Donor selection represented both genders and different ages, as available. Femurs were chosen because they provide the best yield of samples from the same donor. The sample size of 8 specimens per group was chosen in order to fabricate all specimens from the same donor for both the test and control groups. Specimens were machined into 2 mm diameter cortical pins, 23 mm in length. Control samples were cleaned using the standard procedure. Test samples were cleaned with the improved cleaning procedure, with the 1-h H_2O_2 soak. All samples were stored frozen (-70 °C) after cleaning.

Shear testing was conducted using a MTS model 858 servo-hydraulic mechanical test apparatus, equipped with a MTS 2.5 kN static load cell and shear testing fixture built according to ASTM 565–94. The samples were soaked for at least 15 min in normal saline prior to testing. Each sample was loaded to failure at a crosshead speed of 19.1 mm/min (ASTM 2000). The force (load) and displacement data were recorded for each sample. A diagram explaining the experimental design is presented in Figure 2c. The results were analyzed using a two-sample, two-tail student t-test assuming equal variances with statistically significant differences at $p < 0.05$.

In-vivo *tests*

Osteoinductivity

As a second primary goal, it was important to test the effects of hydrogen peroxide treatment on cortical bone osteoinductivity. Femoral cortical bone from donors 16, 17, and 18 (37 Male, 49 Male, 58 Female), after thawing, debriding, and cutting, was cut into 5-mm transverse sections. Control samples were cleaned using the standard procedure. Treatment samples were cleaned with the improved cleaning procedure with a 1-h, a 3-h, or a 5-h H_2O_2 treatment. A negative control group (DBM heat inactivated at 100 °C for 24 h) was also generated, making a total of 13 groups for implantation. After cleaning, the bone was ground into particles between 212 and 850 μm using a Fitzpatrick L1A mill (Fitzpatrick Company, Elmhurst, Il). Bone was then demineralized using 0.6 N hydrochloric acid to a final residual calcium content between 2 and 8%, and mixed with a hyaluronan 4% solution of 700 kD M.W. in a pH 7.2 phosphate buffered saline (LifeCore

Biomedical Inc., Chaska, MN) at a ratio of 8:17 bone to hyaluronan. Next, 15 mg specimens were prepared from each group for transplantation.

The specimens from the 13 groups, were randomized and implanted bilaterally in the hamstring muscles of athymic nude mice ($n = 5$ per group; 65 implants total in 33 mice) (Schwartz et al. 1998), as approved by the University of Medicine and Dentistry of New Jersey Institutional Animal Care and Use Committee (IACUC). Five samples per group were used to detect osteoinduction or the evidence to induce cartilage and then bone formation within the implants. Animals were euthanized at 4 weeks post-implantation. Decalcified histology was performed on the excised samples: 5 histological slides with at least 2 sections per slide were prepared for each sample (at least 10 sections total per sample). Slides were stained with hematoxylin and eosin (H&E) (3 slides per sample) and Masson's trichrome (2 slides per sample) and were evaluated for osteoinductivity. A diagram explaining the experimental design is presented in Figure 2d.

The scoring system described below was used to quantify the relative amount of osteoinduction, with the evaluator blinded to the identification of the implant (Boyan 2000). Osteoinductivity scores were based on the degree to which new bone, bone cells, osteoid, calcified cartilage remnants, and marrow elements were present.

Score	Description
0	No evidence of new bone formation
1	1–25% of the section is covered by new bone
2	26–50% of the section is covered by new bone
3	51–75% of the section is covered by new bone
4	> 75% of the section is covered by new bone

The overall score for each implant was obtained by averaging the highest 5 scores from the histological slides; scores for each experimental group were determined by pooling the overall scores of the individual implants. The results of this scoring are presented as a mean ± standard deviation. Significant differences between groups were determined by the use of the Wilcoxon rank-sum test (Mann–Whitney). p values < 0.05 were considered statistically significant.

Results

Mechanical

The compression data show there are no statistically significant differences in the maximum stress values between the control (standard process) and the test (improved process) groups for all donors in either the longitudinal or transverse tissue orientation for both frozen and freeze-dried

Table 1. The maximum compressive stress and yield stress (MPa) data per donor (Control is the compression specimens subjected to standard process; Test the compression specimens subjected to the improved process. Means are presented with their corresponding standard deviations in parentheses. For all groups $N = 8$. The probability that the means are equal is given as $p(F)$. Values < 0.05 are considered statistically significant. (* = Statistically Significant; Power = 90%). The data demonstrates that the improved process and the standard process are mechanically the same.

Storage	Group	Donor info	Maximum Stress Data			Yield Stress Data		
			Tissue orientation		$p(F)$	Tissue orientation		$p(F)$
			Longitudinal			Longitudinal		
			Control	Test			Control	Test
Frozen	I	39M	164 (7)	159 (9)	0.25	164 (7)	159 (10)	0.27
	II	61F	156 (5)	159 (8)	0.42	156 (5)	159 (8)	0.49
Freeze-Dried	III	39M	219 (27)	222 (27)	0.82	219 (27)	222 (27)	0.81
	IV	61F	206 (27)	202 (38)	0.82	205 (29)	202 (40)	0.83
			Transverse			Transverse		
			Control	Test		Control	Test	
Frozen	V	39M	128 (9)	119 (10)	0.07	96 (8)	92 (10)	0.37
	VI	61F	124 (11)	121 (9)	0.52	87 (4)	80 (6)	0.01 *
Freeze-Dried	VII	39M	153 (20)	167 (25)	0.24	121 (22)	158 (29)	0.01 *
	VIII	61F	127 (15)	117 (15)	0.21	112 (19)	102 (17)	0.29

samples (Table 1). However, there are two statistical differences in the compression yield stress data (Table 1). These occur in Group VI and Group VII, for the 61-year-old female, frozen group, and for the 39-year-old male, freeze-dried group, respectively, both in the transverse tissue orientation. The stress-deformation curves appear typical for human bone for both the longitudinal and transverse compression loading, and the comparable control and test group curves appear similar.

The impact testing data show there are no statistically significant differences in the total kinetic energy values between the control (standard process) and the test (improved process) groups for all donors for both frozen and freeze-dried samples (Table 2). A retrospective power analysis was conducted with alpha = 0.05 and $N = 5$, using the total kinetic energy values. The calculated power (1 - Beta) values are 9, 18, 18, and 12% for Groups IX, X, XI, and XII, respectively.

The shear data show there are no statistically significant differences in the failure stress values between the control (standard process) and the test (improved process) groups for all donors (Table 3).

Table 3. Shear Failure Stress Data per Donor (Control = Shear specimens subjected to Standard process; Test = Shear specimens subjected to the Improved process. Means are presented with their corresponding standard deviations in parentheses. For all groups $N = 8$. The probability that the means are equal is given as $p(t)$. Values < 0.05 are considered statistically significant.) Results show no differences between the control and test groups.

Group	Donor Info	Failure Stress (MPa)		$p(t)$
		Control	Test	
I	21 y/o M	123 (4)	115 (11)	0.08
II	54 y/o F	111 (6)	110 (10)	0.7
III	62 y/o M	106 (8)	106 (11)	0.96
Average		113 (9)	110 (11)	0.23
Min		96	88	
Max		130	130	
N		24	24	

Osteoinductivity

Forty-eight implants were scored for osteoinduction; 8 positive controls; 15 from the 1 h peroxide cleaning groups; 12 from the 3 h peroxide cleaning groups; 9 from the 5 h peroxide cleaning groups; and 4 negative controls. A total of 17 of the 65 implants did not receive an osteoinduction score because the amount of implant found was insufficient for ranking, or, there were artifacts in the slides (positive controls). Eight of these 'unranked' implants were from the 5-h peroxide cleaning group or the negative control; it may be that these implants had significantly resorbed (instead of inducing bone formation) and could not be retrieved.

Hydrogen peroxide cleaning had a statistically significant effect on osteoinductivity, giving a linear decrease with increasing peroxide time (Figure 3). The mean (Standard Deviation) osteoinductivity scores were 3.65 (0.49), 3.04 (0.97), 2.57 (1.36), 1.47 (1.10) for 0, 1, 3, and 5-h H_2O_2 treatment times, respectively. The negative control score was zero. Representative histology photos are shown in Figure 4. Wilcoxon rank-sum test (Mann–Whitney) was used for comparisons. Compared to the control (0 h), the 1-h and the 3-h scores were not significantly different ($p = 0.126$ & $p = 0.068$, respectively), and the 5-h score was significantly different ($p = 0.002$). The trend of decreased osteoinductivity with peroxide cleaning time was consistent for all 3 donors tested (37M, 49M, 58F). The data are presented in Table 4 (raw and pooled osteoinduction scores), and Figure 3 (graph).

Table 2. The Impact Testing Data per Donor, Comparing the Total Kinetic Energy Absorbed At Fracture (Joules) (Control ACF = Impact specimens subjected to standard process; Test ACF = Impact specimens subjected to the improved process. Means are presented with their corresponding standard deviations in parentheses. For all groups $N = 5$. The probability that the means are equal is given as $p(F)$. Values < 0.05 are considered statistically significant. The data does not show a difference between the control and test groups.

Storage	Donor Info	Total Kinetic Energy (Joules)		$p(F)$
		Control ACF	Test ACF	
Frozen	21-M	44.4 (43.3)	32 (12.2)	0.56
	62-M	86.2 (99.6)	36.1 (36.2)	0.32
Freeze-Dried	46-M	40.4 (8.7)	31.2 (17.2)	0.31
	60-M	28 (31.3)	41.6 (24.7)	0.47

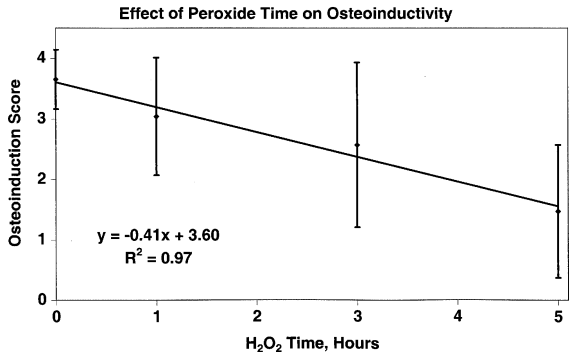

Figure 3. Cortical bone osteoinductivity score verses cleaning time in 3% hydrogen peroxide. Means for each time point are plotted ± Standard deviation. The data shows that as the soak time of cortical bone in peroxide is increased the osteoinductivity in the bone decreases.

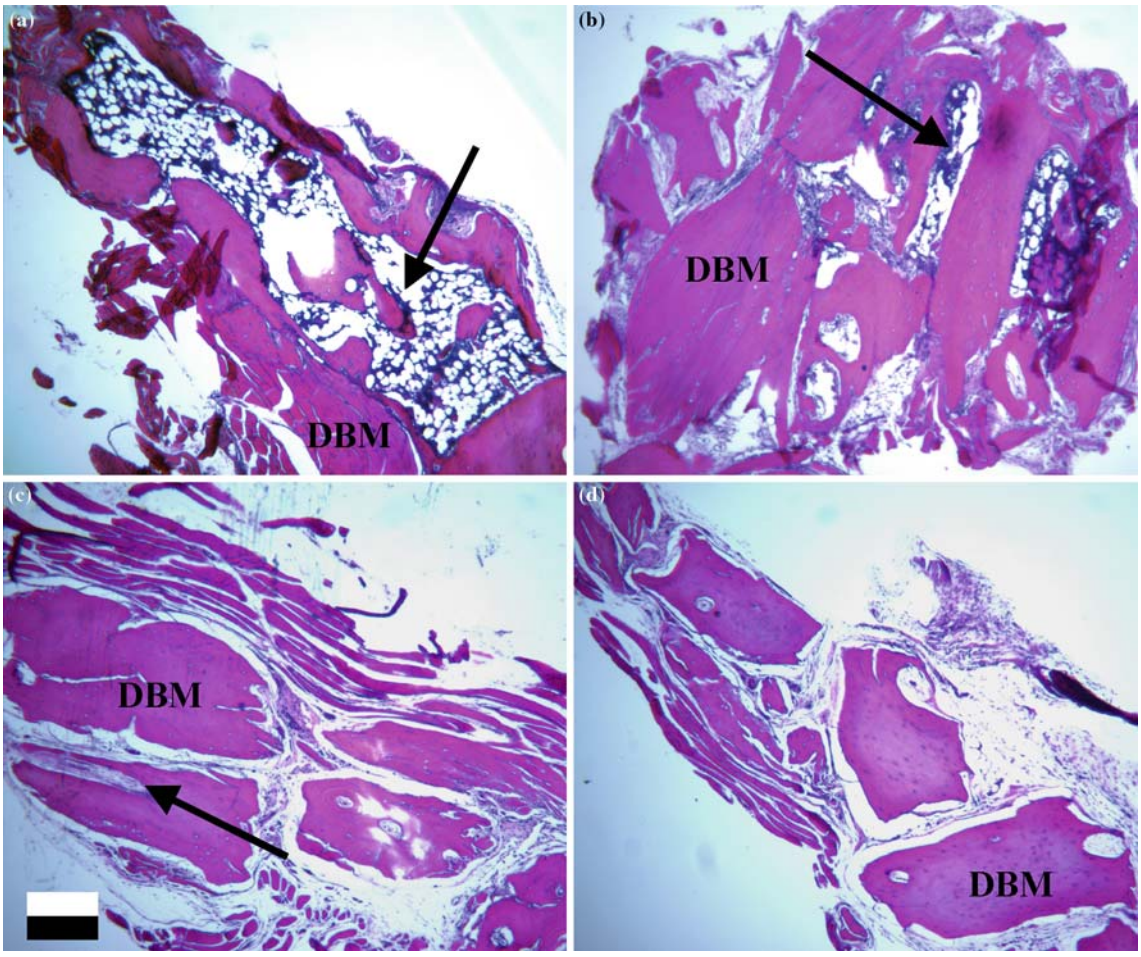

Figure 4. Osteoinduction in response to the various DBM implant groups after 28 days intramuscular implantation in the athymic mouse. BAR = 250 microns. (a) Positive Control DBM. Bone ossicle with new bone and marrow (arrow) associated with residual DBM. (b) DBM treated with hydrogen peroxide for 1 h. Bone ossicle with new bone and marrow (arrow) associated with residual DBM, similar to positive control. (c) DBM treated with hydrogen peroxide for 3 h. Limited new bone formation, with small amount of marrow (arrow) associated with residual DBM. (d) DBM treated with hydrogen peroxide for 5 h. Example of a non-osteoinductive implant; no new bone formation associated with the residual DBM.

Table 4. Osteoinductivity histology scores. Data demonstrates hydrogen peroxide treatment causes a decrease in the osteoinductivity of cortical bone grafts, using a 28-day *in vivo* athymic mouse model.

Treatment	*N*	H_2O_2 Time	Donor Info	Donor Mean	Donor Standard Deviation	Overall Average
H_2O_2 Treated	5	1-h	37M	2.44	1.29	3.04 (0.97)
	5	1-h	49M	3.48	0.79	
	5	1-h	58F	3.20	0.53	
H_2O_2 Treated	5	3-h	37M	3.00	1.41	2.57 (1.36)
	5	3-h	49M	2.05	0.47	
	5	3-h	58F	2.53	2.20	
H_2O_2 Treated	5	5-h	37M	1.00	1.41	1.47 (1.10)
	5	5-h	49M	2.20	1.20	
	6	5-h	58F	1.15	0.87	
Positive Control (No H_2O_2)	4	0-h	37M	3.50	0.71	3.65 (0.49)
	5	0-h	49M	3.67	0.58	
	5	0-h	58F	3.73	0.46	
Negative Control	5	Heat Inactivated	Any/all	0.00	0.00	0.00 (0.00)

Discussion

The goal of this research is to determine whether cortical bone cleaned with an improved cleaning procedure is as safe and effective as the standard procedures used in tissue banking. In the present study, our main goals were to test the effects of hydrogen peroxide treatment on osteoinductivity and mechanical properties of bone. Inherently, hydrogen peroxide is well known to be used for anti-bacterial use, but importantly one needs to determine that the mechanical properties and biological properties (osteoinduction) are not compromised. This research does not address the effects of hydrogen peroxide on cancellous bone.

Initially, the concern was the consequences of using hydrogen peroxide since it is an oxidizing chemical, which therefore could compromise the bone structural proteins. Bone is a naturally anisotropic material, exhibiting different mechanical properties in different directions and experiences combined loading patterns *in vivo*. The loading response of cortical bone requires a variety of testing solutions to accurately characterize the tissue. For this reason, we chose compression testing, impact testing, and shear testing.

No measurable effect was observed on the maximum compressive stress of bone processed with the improved cleaning procedure using an exaggerated 5-h hydrogen peroxide soak, compared to the standard procedure (Table 1). However, two differences were found in the compressive yield stress between samples treated with the improved cleaning procedure (test group) and samples treated with the standard procedure (control group) (Table 1). In the first case, the Group VII data is from transversely cut samples that were freeze-dried. Note, in all cases, freeze-drying nearly triples the standard deviation of the compressive data. The test group yield stress is statistically HIGHER than the control yield stress. This is likely due to natural biological differences in the bone and the introduction of additional variance due to freeze-drying.

The second group with a difference in the compressive yield stress is Group VI (Transverse, Frozen, 61-year-old female) where the difference occurs because the treatment yield stress is LOWER than the control yield stress, at 80 and 87 MPa, respectively. The difference between the groups of 7 MPa was just enough to be statistically significant, using an effect size of 5%, an alpha-level of 0.05, an 80% statistical power level, and $p < 0.05$. The clinical significance of this result is deemed negligible because the corresponding ultimate stress for Group VI was not significantly different.

The analysis of the impact data show there are no statistical differences between the control and improved cleaning treatment groups, using an exaggerated 5-h hydrogen peroxide soak (Table 2). This data does not have the statistical power to detect a significant difference if one truly exists. The variable geometry of the test samples (ACF) and the natural variability in the bone created

variances in the data, greatly reducing the statistical power of this data. A retrospective power analysis revealed that the sample size would need to be increased to greater than 35 samples per group to obtain statistically relevant data; this is not possible because insufficient bone occurs in a single donor for this sample size.

There was no measurable effect of the improved cleaning procedure on the failure stress of cortical bone in shear (Table 3). The average failure stress of the test samples is slightly lower than that of the control samples, but the difference is not statistically significant. The standard deviations of the control and test samples are similar.

The improved cleaning procedure does not affect the mechanical strength of cortical bone allografts. In addition, it is noteworthy that the longitudinal ultimate compressive strength values of 164 ± 7 MPa, 159 ± 9 MPa obtained for the 39 year old male frozen samples in this study are similar to values reported in the literature of 170 MPa ± 4.3 for human (20–39 years) femur (Fung 1993).

Another concern was the consequences of using hydrogen peroxide because it could potentially compromise bone morphogenetic proteins (BMP's) and the osteoinductivity of a graft. BMP's are low molecular weight polypeptides that regulate osteoinduction (Wolfe et al. 1999). Positive control samples showed excellent osteoinductivity, with an average score of 3.65 on a scale of 0–4. Hydrogen peroxide cleaning caused a statistically significant decrease in osteoinductivity with increasing time. After 5 h of hydrogen peroxide treatment in the temperature controlled ultrasonic bath, the osteoinductivity score was statistically decreased from 3.65 in the positive control to 1.47 ($p = 0.002$). The trend of decreased osteoinductivity was consistent for all three donors (Table 4 and Figure 3). It was for this reason that the hydrogen peroxide step of the improved cleaning procedure was limited to a 1-h soak. The improved cleaning procedure, with a 1-h hydrogen peroxide soak, does not significantly affect the osteoinductivity of cortical bone allografts.

The results demonstrate the strength of a cortical bone graft is maintained and there is no significant loss in osteoinductivity with 1-h of peroxide treatment. Hence, we accept the null hypothesis that the improved cleaning procedure and the standard procedure are equivalent.

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References

Akkus O. and Rimnac C.M. 2001. Fracture resistance of gamma radiation sterilized cortical bone allografts. J. Orthop. Res. 19: 927–934.

American Association of Tissue Banks 2004. 2002 Annual Survey of Accredited Tissue Banks. American Association of Tissue Banks, McLean, Virginia, pp. 20

Aspenberg P., Johnsson E. and Thorngren K.G. 1990. Dose-dependent reduction of bone inductive properties by ethylene oxide. J. Bone Joint Surg. Br. 72: 1036–1037.

ASTM 2000. Standard Test Method for Shear Testing of Aluminum and Alluminum Alloy Rivets and Cold Heading Wire and Rods. ASTM, West Conshohocken, PA, pp. 1–3.

Boyan B. 2000. Draft Standard: Standard Guide for the Assessment of Bone Inductive Materials (ASTM). pp.6.

Boyce T., Edwards J. and Scarborough N. 1999. Allograft bone. The influence of processing on safety and performance. Orthop. Clin. North Am. 30: 571–581.

Currey J.D., Foreman J., Laketic I., Mitchell J., Pegg D.E. and Reilly G.C. 1997. Effects of ionizing radiation on the mechanical properties of human bone. J. Orthop. Res. 15: 111–117.

Fung Y.C. 1993. Biomechanics Mechanical Properties of Living Tissues. 2nd ed. Springer-Verlag, New York.

Hallfeldt K.K., Stutzle H., Puhlmann M., Kessler S. and Schweiberer L. 1995. Sterilization of partially demineralized bone matrix: the effects of different sterilization techniques on osteogenetic properties. J. Surg. Res. 59: 614–620.

Hamer A.J., Stockley I. and Elson R.A. 1999. Changes in allograft bone irradiated at different temperatures. J. Bone Joint Surg. Br. 81: 342–344.

Head W.C. and Malinin T.I. 2000. Results of onlay allografts. Clin. Orthop. 371: 108–112.

Ijiri S., Yamamuro T., Nakamura T., Kotani S. and Notoya K. 1994. Effect of sterilization on bone morphogenetic protein. J. Orthop. Res. 12: 628–636.

Ito T., Sakano S., Sato K., Sugiura H., Iwata H., Murata Y. and Seo H. 1995. Sensitivity of osteoinductive activity of demineralized and defatted rat femur to temperature and duration of heating. Clin. Orthop.: 267–275.

Kummer F.J., Chen D. and Spivak J.M. 1998. Optimal selection and preparation of fresh frozen corticocancellous allografts for cervical interbody spinal fusion. Spine 23: 2295–2298.

Mankin H.J. 1993. Current status of allografting. Pttsburgh Orthop. J. 4: 114–120.

Mitchell E.J., Stawarz A.M., Kayacan R. and Rimnac C.M. 2004. The effect of gamma radiation sterilization on the fatigue crack propagation resistance of human cortical bone. J. Bone Joint Surg. Am. 86(A): 2648–2657.

Mow V.C. and Hayes W.C. 1991. Basic Orthopaedic Biomechanics. Raven Press, New York.

Munting E., Wilmart J.F., Wijne A., Hennebert P. and Delloye C. 1988. Effect of sterilization on osteoinduction. Comparison of five methods in demineralized rat bone. Acta Orthop. Scand. 59: 34–38.

Phillips G.O. and Morales J. 2002. Catalysts for Better Health Care: Medical Tissue Banks Bring Multiple Benefits to Countries. In IAEA Bulletin, pp. 17–20.

Schwartz Z., Somers A., Mellonig J.T., Carnes D.L.Jr., Dean D.D., Cochran D.L. and Boyan B.D. 1998. Ability of commercial demineralized freeze-dried bone allograft to induce new bone formation is dependent on donor age but not gender. J. Periodontol. 69: 470–478.

Speirs A.D., Hotz M.A., Oxland T.R., Hausler R. and Nolte L.P. 1999. Biomechanical properties of sterilized human auditory ossicles. J. Biomech. 32: 485–491.

Thoren K. and Aspenberg P. 1995. Ethylene oxide sterilization impairs allograft incorporation in a conduction chamber. Clin. Orthop. 318: 259–264.

Tomford W.W. and Mankin H.J. 1999. Bone banking: update on methods and materials. Orthop. Clin. North Am. 30: 565–570.

U.S. Government 2002. Code of Federal Regulations, Title 27 Alcohol, Tobacco, and Firearms, Part 21 Formulas for Denatured Alcohol and Rum, Section 37 Formula No. 3-C.

Wolfe M.W., Salkeld S.L. and Cook S.D. 1999. Bone morphogenetic proteins in the treatment of non-unions and bone defects: historical perspective and current knowledge. Univ. Penn. Orthop. J. 12: 1–6.

Woll J.E. and Kasprisin D. (eds) 2001. Standards For Tissue Banking. American Association of Tissue Banks, McLean VA.