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Basic Research

Deep-Freezing Temperatures During Irradiation Preserves the Compressive Strength of Human Cortical Bone Allografts: A Cadaver Study

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Abstract

Background Gamma irradiation, which minimizes the risk of infectious disease transmission when human bone allograft is used, has been found to negatively affect its biomechanical properties. However, in those studies, the deep-freezing temperature during irradiation was not necessarily maintained during transportation and sterilization, which may have affected the findings. Prior reports have also suggested that controlled deep freezing may mitigate

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the detrimental effects of irradiation on the mechanical properties of bone allograft.

Question/purpose Does a controlled deep-freezing temperature during irradiation help preserve the compressive mechanical properties of human femoral cortical bone allografts?

Methods Cortical bone cube samples, each measuring 64 mm^3, were cut from the mid-diaphyseal midshaft of five fresh-frozen cadaver femurs (four male donors, mean [range] age at procurement 42 years [42 to 43]) and were allocated via block randomization into one of three experimental groups (with equal numbers of samples from each donor allocated into each group). Each experimental group consisted of 20 bone cube samples. Samples irradiated in dry ice were subjected to irradiation doses ranging from 26.7 kGy to 27.1 kGy (mean 26.9 kGy) at a deep-freezing temperature below -40°C (the recommended long-term storage temperature for allografts). Samples irradiated in gel ice underwent irradiation doses ranging from 26.2 kGy and 26.4 kGy (mean 26.3 kGy) in a freezing temperature range between -40°C and 0°C. Acting as controls, samples in a third group were not subjected to gamma irradiation. The mechanical properties (0.2% offset yield stress, ultimate compression stress, toughness, and the Young modulus) of samples from each group were subsequently evaluated via axial compression loading to failure along the long axis of the bone. The investigators were blinded to sample group during compression testing.

Results The mean ultimate compression stress (84 ± 27 MPa versus 119 ± 31 MPa, mean difference 35 [95% CI 9 to 60]; p = 0.005) and toughness (3622 ± 1720 kJ/m^3 versus 5854 ± 2900 kJ/m^3, mean difference 2232 [95% CI 70 to 4394]; p = 0.009) of samples irradiated at a higher temperature range (-40°C to 0°C) were lower than in those irradiated at deep-freezing temperatures (below -40°C). The mean 0.2% offset yield stress (73 ± 28 MPa versus 109 ± 38 MPa, mean difference 36 [95% CI 11 to 60]; p = 0.002) and ultimate compression stress (84 ± 27 MPa versus 128 ± 40 MPa, mean difference 44 [95% CI 17 to 69]; p < 0.001) of samples irradiated at a higher temperature range (-40°C to 0°C) were lower than the nonirradiated control group samples. The mean 0.2% offset yield stress (73 ± 28 MPa versus 101 ± 28 MPa, mean difference 28 [95% CI 3 to 52]; p = 0.02; effect size = 1.0 [95% CI 0.8 to 1.2]) of samples irradiated at higher temperature range (-40°C to 0°C) were no different with the numbers available to those irradiated at deep-freezing temperature. The mean toughness (3622 ± 1720 kJ/m^3 versus 6231 ± 3410 kJ/m^3, mean difference 2609 [95% CI 447 to 4771]; p = 0.02; effect size = 1.0 [95% CI 0.8 to 1.2]) of samples irradiated at higher temperature range (-40°C to 0°C) were no different with the numbers available to the non-irradiated control group samples. The mean 0.2% offset yield stress, ultimate compression stress, and toughness of samples irradiated in deep-freezing temperatures (below -40°C) were not different with the numbers available to the non-irradiated control group samples. The Young modulus was not different with the numbers available among the three groups.

Conclusion In this study, maintenance of a deep-freezing temperature below -40°C, using dry ice as a cooling agent, consistently mitigated the adverse effects of irradiation on the monotonic-compression mechanical properties of human cortical bone tissue. Preserving the mechanical properties of a cortical allograft, when irradiated in a deep-freezing temperature, may have resulted from attenuation of the deleterious, indirect effects of gamma radiation on its collagen architecture in a frozen state. Immobilization of water molecules in this state prevents radiolysis and the subsequent generation of free radicals. This hypothesis was supported by an apparent loss of the protective effect when a range of higher freezing temperatures was used during irradiation.

Clinical Relevance Deep-freezing temperatures below -40°C during gamma irradiation may be a promising approach to better retain the native mechanical properties of cortical bone allografts. A further study of the effect of deep-freezing during gamma radiation sterilization on sterility and other important biomechanical properties of cortical bone (such as, tensile strength, fracture toughness, and fatigue) is needed to confirm these findings.

Introduction

Gamma irradiation has been reported to have negative effects on the monotonic biomechanical properties of bone allografts, particularly by decreasing its yield stress, ultimate compression stress, resilience [18, 21], and toughness [9, 19]. Concerns regarding these adverse effects continue to be a source of apprehension in its clinical application [28, 34, 36]. Radiation as a terminal sterilization method diminishes the risk of donor-to-recipient disease transmission through its pathogen-killing effects [33, 49]. It acts via direct and indirect effects [48]. The direct effect occurs when a direct transfer of radiation energy interrupts the polypeptide bonds in the pathogen's nucleus and organelles, leading directly to death. The indirect effect, conversely, is the ability of water radiolysis to produce free radicals, which initiates a chain reaction that ultimately results in DNA damage, deterioration in enzymatic and structural protein function, and altered membrane permeability. This indirect effect is the more dominant of the two effects, and its efficacy is amplified by the presence of oxygen, water, and a higher temperature at the time of irradiation [10, 11, 13, 33, 34, 43].

The destructive effects of gamma irradiation are unfortunately not confined to the microorganisms it seeks to

eliminate. The undesired negative effects of irradiation on the mechanical properties of allograft bone primarily arise from its action on the collagen matrix. Physical, non-enzymatic collagen molecule scission and formation of weak, immature collagen cross-linkages are thought to induce brittleness [8, 10, 42]. This effect is dose-dependent, whereby an increase in the radiation dose leads to degradation in the biomechanical properties of allograft bone [2, 8, 9, 34, 42]. However, several studies have disagreed about the effect of temperature during irradiation on the mechanical properties of bone allografts [1, 9, 18, 19, 21]. Because the temperature during irradiation differs between these studies, as does the extent to which temperature is regulated during the transportation and irradiation processes, direct comparisons are further limited [18, 19, 21], although as per the recommendation of the American Association of Tissue Banks Standards [37], bone allograft must be maintained below -40°C during processing, transit, and storage. Thus, there does not appear to be consensus about the effect of different freezing temperatures during irradiation on the mechanical behavior of human cortical bone allograft.

We therefore asked: Does a controlled deep-freezing temperature during irradiation help preserve the compressive mechanical properties of human femoral cortical bone allografts?

Materials and Methods

Study Subjects

The experiment was conducted using femurs from human cadavers. We procured bones in accordance with the standards of the Asia Pacific Association of Surgical Tissue Banking. Only bones that were unsuitable for implantation, that is, those with negative serology results but with positive initial swab culture results, were used. Procured bones were packaged, labeled, and stored in our bone bank's freezer at -75°C ± 10°C.

Description of Experiment, Treatment, or Surgery

Five femurs from four male donors were used in this experiment; no donors had apparent premortem bone-related disorders. Three were left-sided and two were right-sided. The mean (range) age at the time of procurement was 42 years (42 to 43). Each femur was unpackaged, labeled, thawed at room temperature, rinsed, and rehydrated with normal saline for 30 minutes before sample preparation. All residual soft tissue was excised. The diaphysis of each femur was measured and cut perpendicular to the long axis using a table-mounted band saw (JG210A, Orimas) into four equal cortical segments. For homogeneity, only the midshaft segments, that is, segments 2 and 3, were used (Fig. 1). A single cortical ring was cut from the mid-diaphyseal ends of these two segments, producing two cortical rings that were the most representative of the midshaft of each femoral bone. To account for the anisotropic properties of bone [2, 19 46, 50], we marked the inferior surfaces of the rings with a permanent marker to identify their axial anatomic orientation throughout processing and especially during mechanical testing. The bone rings were successively cut into eight sections and attached to a custom-made cutting jig. Then they were carefully run through the band saw to attain the desired products: bone cubes of approximately 64 mm^3 (Fig. 1). Hydration of each sample was maintained throughout the machining process. Final measurements of each cube were then confirmed twice with a digital caliper (CD-6), and each was packaged separately and labeled.

Eighty cubes in total were obtained from five femurs; nine cubes did not meet the desired measurements and were excluded. Using a computer-generated block randomization technique, we randomized 12 samples from each of the five femurs equally into three experimental groups (total number of cubes = 60, samples per experimental group = 20): (1) radiated in dry ice for irradiation below -40°C (that is, deep-freezing temperature), (2) irradiated in gel ice for irradiation between -40°C and 0°C (that is, freezing temperature), and (3) control samples, which were not irradiated. All samples in each group were packaged together and stored again at -75°C ± 10°C. The mean volumes of samples radiated in gel ice, radiated in dry ice, and the control group were normally distributed, with no differences in the mean cube volumes among the three groups: (1) irradiated in gel ice versus dry ice (70.28 ± 2.77 mm^3 versus 70.40 ± 3.3 mm^3, mean difference 0.12 [CI -2.27 to 2.50]; p = 0.99), (2) irradiated in gel ice versus non-irradiated control (70.28 ± 2.77 mm^3 versus 70.33 ± 3.08 mm^3, mean difference 0.05 [CI -2.34 to 2.43]; p = 0.99), (3) irradiated in dry ice versus non-irradiated control (70.40 ± 3.3 mm^3 versus 70.33 ± 3.08 mm^3, mean difference 0.07 [CI -2.31 to 2.47]; p = 0.99).

Samples in the irradiated groups (irradiated in dry ice and irradiated in gel ice) received irradiation treatment before mechanical testing. Gamma irradiation was performed at the Malaysian Nuclear Agency, Bangi, Selangor, Malaysia. A calibrated thermocouple thermometer probe (Ebro EBI310, Xylem Analytics) was secured to the plastic bag containing the samples of the irradiated in dry ice group, immediately after their removal from storage at -75°C ± 10°C. The bag was placed in a polystyrene foam box containing 20 slabs of dry ice. The box was sealed and transported to the irradiation facility, with the temperature in the box logged throughout the journey. The temperature probe, which was not radiation-compatible, was disconnected immediately before its entry into the irradiation loop.

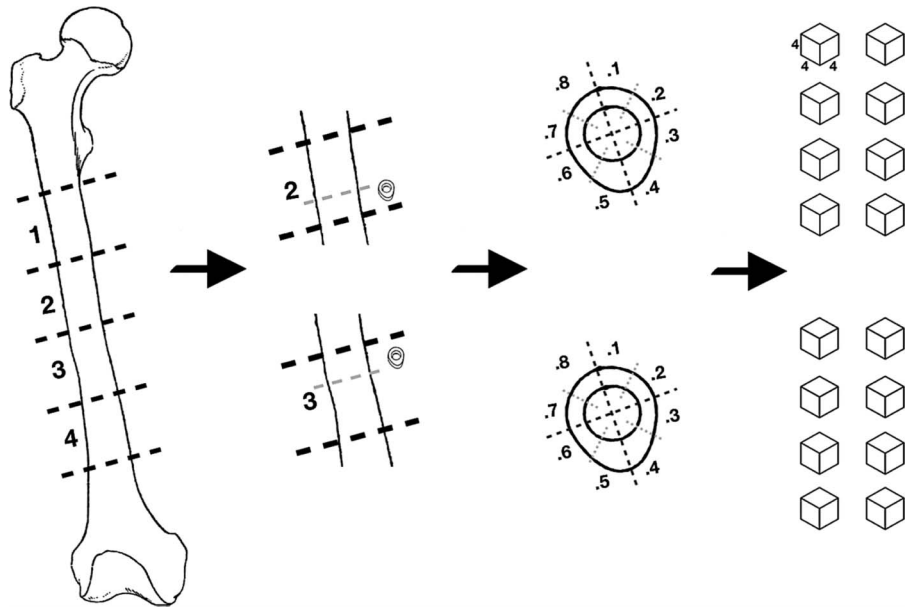

Fig. 1 This schematic diagram shows the bone cutting process. The five femurs were systematically cut from segments into cortical rings, then into quarters, and finally into 64-mm^3 cubes.

The irradiated in dry ice and irradiated in gel ice samples were subjected to a minimum sterilization dose of 25 kGy to achieve a sterility assurance level of 10^{-6}. The minimum and maximum doses in the group irradiated in dry ice were 26.7 kGy and 27.1 kGy (mean 26.9 kGy), respectively. The minimum and maximum doses in the irradiated in gel ice group were 26.2 kGy and 26.4 kGy (mean 26.3 kGy), respectively. The duration of irradiation for the samples irradiated in dry ice and in gel ice were 11.5 hours and 12 hours, respectively.

The polystyrene foam box was collected after irradiation, and the temperature probe was immediately reattached to the samples. After being returned to the bone bank, the samples were returned to storage at -75°C ± 10°C. Using linear trend estimation and gradient analysis, we determined the temperatures in the irradiation interval. The rationale for this approach is that gamma irradiation is a cold process that does not generate meaningful increases in temperature in the irradiation field, therefore making internal temperature fluctuation highly unlikely [10, 15, 22, 33, 43]. This process was repeated for the samples irradiated in gel ice, but the polystyrene foam box was filled with 28 bags of gel ice (Thermafreeze) as the cooling material instead. Each of these powder-based gel ice packs were first soaked in water until fully expanded before storage at -75°C ± 10°C for 3 days before its use. The specified amount of cooling material was based on a validation study at our institution [40] that examined the ability of composites of dry and gel ice to maintain subzero temperatures (in a polystyrene foam box like that used in the current experiment) for allograft transportation and irradiation (Supplementary Table 1; http://links.lww.com/CORR/A626).

Samples from the group irradiated in dry ice were simultaneously irradiated with a target temperature of below -40°C. The mean temperature throughout the irradiation period of 11.5 hours was -78.4°C ± 0.2°C, with a range of -78.6°C to -78.1°C; as anticipated, using 20 slabs of dry ice as the cooling material for the irradiated in dry ice group allowed for an extremely consistent, stable, and predictable temperature range (Fig. 2).

Unlike in the irradiated in dry ice group, the temperature for the irradiated in gel ice group steadily increased over time. To ensure irradiation occurred within the intended temperature range, a waiting interval between packaging and entry into the radiation loop was necessary. Consistent with the results of the previous validation study [40], the waiting time between the packaging and the increase in temperature above -40°C was approximately 6 hours. The mean (range) temperature throughout the irradiation period of 12 hours was -13.4°C ± 7.8°C (-29°C to -2.9°C) (Fig. 3).

Variables, Outcome Measures, Data Sources, and Bias

Biomechanical testing was performed in the Biomechanics Laboratory of the University of Technology Malaysia. The samples were transported in dry ice (maintaining an

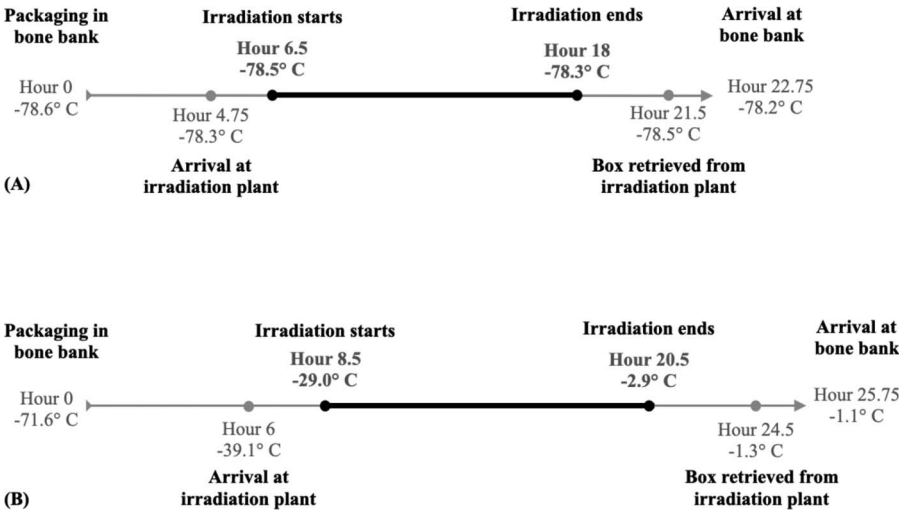

Fig. 2 A-B This figure shows timelines of the irradiation process for the (**A**) irradiated in dry ice and (**B**) irradiated in gel ice samples. The temperatures at key points are noted, with time (in hours) expressed as the cumulative duration starting after the packaging process at Hour 0. Periods of irradiation are highlighted for both treatment groups.

internal temperature of below -40°C throughout), allowed to thaw at room temperature on arrival, and were rinsed thoroughly with normal saline solution. An Instron 8874 Axial-Torsion System (Instron Co) machine with a maximum load capacity of 25 kN was used for mechanical testing. To offset the effect of machine compliance [50], the system was calibrated by conducting an initial loading cycle without any specimen in place. In each subsequent cycle, a well-hydrated allograft cube was loaded (marked inferior-side down) on the universal testing machine between its two platform platens, preloaded to 10 N, and subjected to compressive axial load to failure along its long axis, for which displacements were recorded (Fig. 4). For each sample, the adjustable grips were modified and aligned to ensure an even distribution of compressive load. The strain rate was kept at 0.001 s^{-1}. The investigators (AS, APMS [who was not a study author]) performing the mechanical testing were blinded to the assigned sample groups. Load-displacement data were replotted as a stress-strain curve for each sample. Strain was automatically calculated by Instron Bluehill (Instron Co) software. The resultant values were verified manually with the following equation: $\varepsilon = \Delta L/L$, where ε = engineering normal strain, ΔL = change in length of sample, and L = original length of sample. Four biomechanical parameters were extracted: 0.2% offset yield stress, ultimate compressive stress, Young modulus, and toughness. The yield stress, which indicates the limit of a sample's elastic behavior and the beginning of its plastic behavior, was determined using the 0.2% offset method [4, 32, 45, 46]. Ultimate compressive stress, defined as the maximum amount of compressive stress a material can resist without fracturing, was taken as the largest strain value of each stress-strain curve. The Young modulus is a measure of the resistance of a material to elastic deformation under load and was derived by analyzing the gradient of the steepest slope of the linear portion of the 0.2% offset stress-strain curve, where stress is proportional to strain; the nonlinear toe-region was always excluded from this analysis. Toughness of a material, defined as its ability to absorb maximum energy before the point of fracture, was calculated as the area under the entire stress-strain curve, limited on the x-axis by the point of fracture (Fig. 5).

Ethical Approval

Ethical approval was obtained from the University Malaya Medical Centre's ethics committee (approval number 1037.8).

Statistical Analysis, Study Size

All data were analyzed with Excel (Microsoft) and SPSS 20 (IBM) software. Data from a pilot study were used as reference for calculating the sample size; a total of 20 samples per group (in three equal-sized groups) were required to achieve a power of 95% with a 5% level of significance. The normality of distribution for each data set was first established via the Shapiro-Wilk test. The 0.2% offset yield stress, ultimate compressive stress, and toughness data set were found to be

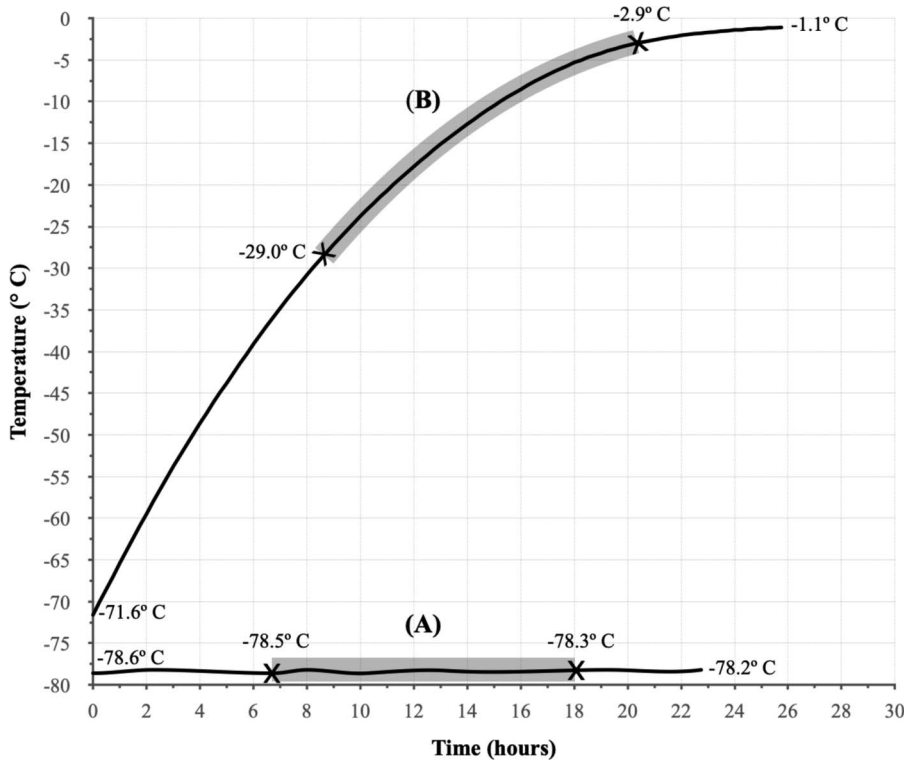

Fig. 3 Temperature pattern graphs are shown for (**A**) the irradiated in dry ice and (**B**) the irradiated in gel ice samples. All samples of each group were irradiated simultaneously. Periods of irradiation are highlighted for both groups, with Xs indicating the beginning and end of the respective periods. The internal temperature for the irradiated in dry ice samples was consistent throughout the process until unpackaging at 23 hours, with a mean temperature during irradiation of -78.4°C ± 0.2°C. The temperature in the irradiated in gel ice group, however, showed a steady but nonlinear increase from an initial temperature of -71.6°C to -1.1°C at 26 hours, with a mean temperature during irradiation of -13.4°C ± 7.8°C.

normally distributed and were subjected to the parametric one-way ANOVA test. When significance was found, a Bonferroni post hoc test was conducted to identify any difference among the three groups. Considering three groups, the adjusted p value based on the Bonferroni method was 0.05 / 3 = 0.0167. A p value < 0.0167 was therefore considered statistically significant. The Young modulus data set was found not to be normally distributed and was subjected to the nonparametric Kruskal-Wallis test. To quantify and compare the magnitude of effect between each treatment group, the effect size was calculated using the Cohen *d* method.

Results

Allografts irradiated in gel ice demonstrated lower mean 0.2% offset yield stress than those that were non-irradiated (73 ± 28 MPa versus 109 ± 38 MPa, mean difference 36 [95% CI 11 to 60]; p = 0.002; effect size = 1.1 [95% CI 0.9 to 1.3]) but were no different with numbers available with those irradiated in dry ice (73 ± 28 MPa versus 101 ± 28 MPa, mean difference 28 [95% CI 3 to 52]; p = 0.02; effect size = 1.0 [95% CI 0.8 to 1.2]). Allografts irradiated in gel ice also demonstrated lower mean ultimate compressive stress when compared with those that were non-irradiated (84 ± 27 MPa versus 128 ± 40 MPa, mean difference 44 [95% CI 17 to 69]; p < 0.001; effect size = 1.3 [95% CI 1.1 to 1.5]) and with those irradiated in dry ice (84 ± 27 MPa versus 119 ± 31 MPa, mean difference 35 [95% CI 9 to 60]; p = 0.005; effect size = 1.2 [95% CI 1.0 to 1.4]). Allografts irradiated in gel ice also demonstrated lower mean toughness when compared with those irradiated in dry ice (3622 ± 1720 kJ/m^3 versus 5854 ± 2900 kJ/m^3, mean difference 2232 [95% CI 70 to 4394]; p = 0.009; effect size = 0.9 [95% CI 0.7 to 1.1]) but were not different with numbers available when compared to those which were non-irradiated (3622 ± 1720 kJ/m^3 versus 6231 ± 3410 kJ/m^3, mean difference 2609 [95% CI 447 to 4771];

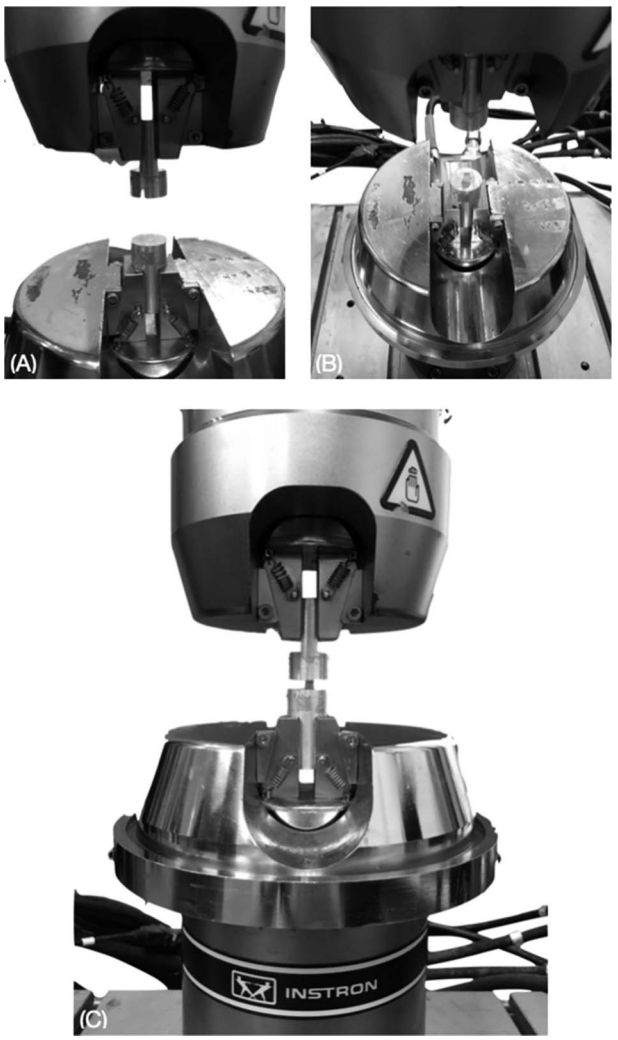

Fig. 4 A-C These photographs show the compression testing set-up. (**A**) In the mechanical testing process, compression platens were secured to the adjustable upper and lower grips of the Instron and (**B**) an allograft cortical bone cube was placed on the lower plate. (**C**) Axial force generated by the load cell compressed the specimen to failure.

p = 0.02; effect size = 1.0 [95% CI 0.8 to 1.2]). There was no difference in mean 0.2% offset yield stress (101 ± 28 MPa versus 109 ± 38 MPa, mean difference 8 [95% CI -17 to 32]; p > 0.99; effect size = 0.2 [95% CI 0.0 to 0.4]), ultimate compressive stress (119 ± 31 MPa versus 128 ± 40 MPa, mean difference 9 [95% CI -17 to 34]; p > 0.99; effect size = 0.3 [95% CI 0.1 to 0.5]), and toughness (5854 ± 2900 kJ/m^3 versus 6231 ± 3410 kJ/m^3, mean difference 377 [95% CI -1785 to 2539]; p = 0.74; effect size = 0.1 [95% CI -0.1 to 0.3]) between samples irradiated in dry ice and those in the control group (Fig. 6). There was no difference in the median Young modulus among the three groups: (1) irradiated in gel ice versus irradiated in dry ice (2.80 [2.17] GPa versus 3.51 [2.6] GPa, difference of medians 0.71 [95% CI 0.52 to 1.00]; p = 0.21; (2) irradiated in gel ice versus non-irradiated control (2.8 [2.17] GPa versus 3.49 [2.03] GPa, difference of medians 0.81 [95% CI 0.54 to 1.03]; p = 0.21); and (3) irradiated in dry ice versus non-irradiated control (3.51 [2.6] GPa versus 3.49 [2.03] GPa, difference of medians -0.02 [95% CI -0.24 to 0.2]; p = 0.21) (Table 1).

Discussion

Infection remains a considerable problem associated with the clinical application and safety of musculoskeletal allografts [12, 29, 36]. Gamma irradiation is a reliable and popular method of sterilization; it is highly penetrative (resulting in a more uniform dose distribution, even in solid matter) [11, 28], leaves no harmful toxic residue [6, 25, 38], does not generate temperature in the irradiation field [10, 15, 22, 33, 43], and it simplifies the packaging and processing procedures because of the nature of terminal sterilization [5]. Its most apparent deficiency is its deleterious effects on the mechanical properties of these allografts. In this study, we observed that the static, compressive mechanical properties of allograft cortical bone tissue irradiated at a deep-freezing temperature range below -40° C were not worse than those that were nonirradiated (fresh-frozen). This effect did not extend to samples irradiated at a freezing temperature range of -40° C to 0° C; there were decreases in three of the four outcome measures (0.2% offset yield stress, ultimate compressive stress, and toughness) for this group compared with the deep freezing and control groups when effect size was considered.

Limitations

This study has several limitations. First, only femurs from five age- and sex-matched donors were available for this experiment. A larger number of allografts and an evaluation of bone from males and females [26], if available, would permit greater observation of possible variations in donor bone. Working with limited resources, the inclusion of two femurs from one of the donors was inevitable. However, as each femur had contributed an equal number of samples into each experimental group by virtue of block randomization, we believe that this mitigated potential confounding effects. Second, although our results point to an apparent deterioration in the compressive mechanical properties of allograft irradiation when irradiated at a higher temperature range of -40°C to 0°C, our study lacked a positive control experimental group. The inclusion of an experimental group receiving irradiation at ambient temperature [1, 9, 18, 19, 21] would have allowed for these additional comparisons to be made. Also, we considered only monotonic compression; an

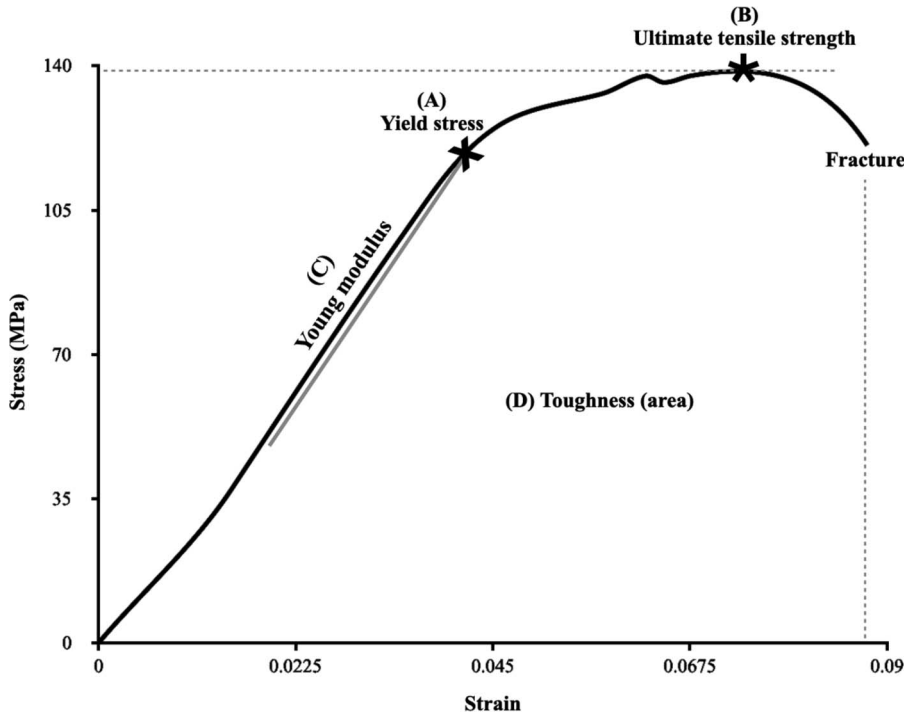

Fig. 5 This graph shows the stress-strain curve of a sample from the control group. (**A**) The yield stress (x), which indicates the limit of a sample's elastic behavior and the beginning of its plastic behavior, was determined using the 0.2% offset method [4, 32, 45, 46]. (**B**) Ultimate compressive stress (asterisk), defined as the maximum amount of compression stress a material can resist without fracturing, was taken as the largest stress of each stress-strain curve. (**C**) The Young modulus was derived by analyzing the gradient of the steepest slope of the linear portion of the stress-strain curve, where the stress was proportional to the strain; the nonlinear toe region was excluded from analysis. (**D**) The toughness of a material, defined as its ability to absorb the maximum energy before the point of fracture, was calculated as the area under the entire stress-strain curve, limited on the x-axis by the point of fracture; MPa = megapascal.

evaluation of monotonic tension data would help to confirm our findings. Other noteworthy mechanical parameters affected by gamma irradiation that have been studied but not incorporated here include an assessment of the viscoelastic properties [30, 47], physiologic cyclic fatigue life [20], fracture toughness [1], and the fatigue crack propagation resistance of bone [31]. We note the paucity of description of temperature regulation during irradiation in existing reports on these mechanical properties. As allograft failures can occur well below the yield stress either because of a stress concentration or due to repetitive stress, these parameters are relevant to the application and long-term performance of allografts in clinical practice; perhaps the methods employed here for the most elementary of monotonic characterization, that is, compression testing, may serve as a reference for future work in that direction.

The inclusion of a biochemical and microscopic evaluation of samples after irradiation and mechanical testing would also allow for a more quantitative and qualitative assessment of the effects of different freezing temperatures during irradiation on the microstructure of the collagen matrix [7, 9, 18, 24, 41], but was beyond the scope and feasibility of this study. Conventionally, collagen content and extent of cross-linkage are evaluated via enzymatic digestion with or without liquid chromatography of irradiated samples [7, 18, 41], while the presence of microcracks is assessed histologically [1, 9]. Finally, as our current experiments were conducted using a standard minimum irradiation dose of 25 kGy without an accompanying study of pathogen inactivation, we did not verify the extent to which the sterilization process was effective. Although deep-freezing temperatures might protect the mechanical properties of irradiated bone allografts, there is a major concern about the attenuation of the effects of irradiation on the inactivation of pathogens (particularly the HIV virus) at conventional irradiation doses. Available reports suggest that higher radiation dosages may be required for virus inactivation at lower radiation

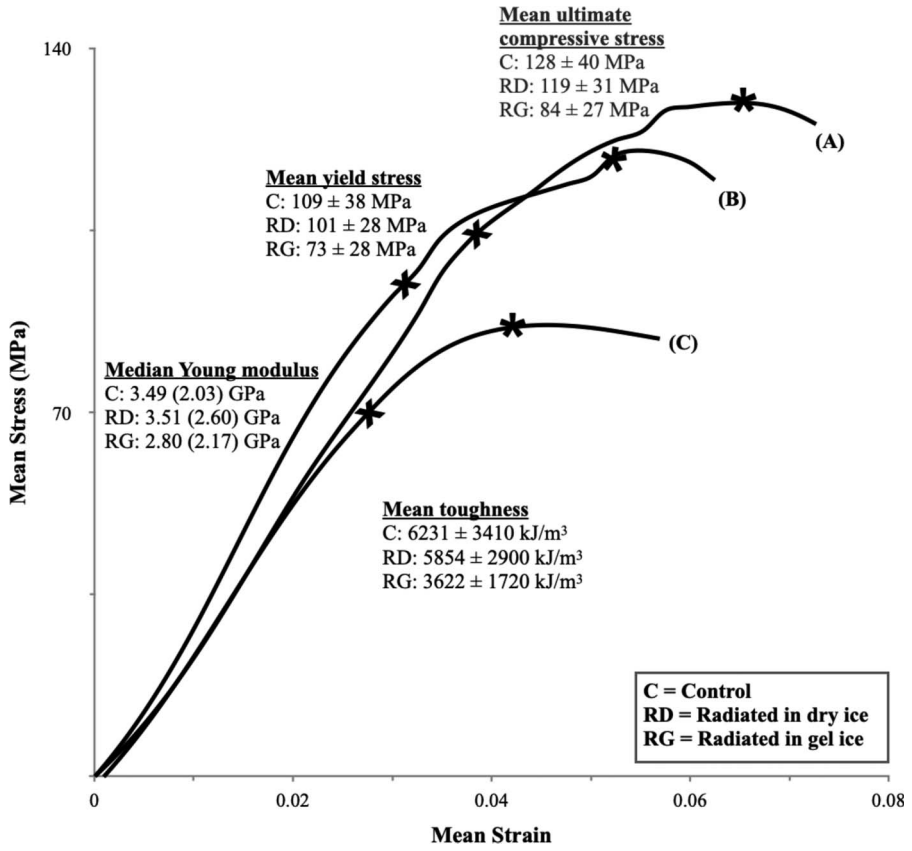

Fig. 6 These graphs show the stress-strain curves plotted using the combined mean of stress and strain values of every sample (taken at intervals of one second) of the (**A**) control, (**B**) irradiated in dry ice, and (**C**) irradiated in gel ice experimental groups. The mean yield stress (x), ultimate compressive stress (asterisk), the Young modulus, and toughness of each experimental groups are shown; MPa = megapascal; GPa = gigapascal; kJ/m^3 = kilojoule per cubic meter.

temperatures [14, 17, 39], which therefore makes a concurrent evaluation of mechanical properties along with validation of sterility at different radiation dosages essential.

Influence of Temperature on Compressive Strength

Our findings were like those of Hamer et al. [18]. As opposed to their work, we stringently monitored temperature during the transportation and irradiation process. In addition, we included a non-irradiated group as a control, and another sample group which was irradiated at higher freezing temperature (-40°C to 0°C) instead of at room temperature. Our results, however, agree with their hypothesis that the protective effects of deep-freezing temperatures during irradiation may be due to the sequelae of water molecules that are immobilized while frozen; with the failure of water molecules to participate in the formation of free radicals via radiolysis, the negative effects of radiation on the architecture of collagen are attenuated. In our study, this protective effect was only apparent at deep freezing temperatures, or below -40°C, and not when the temperatures were maintained between -40°C to 0°C. Another possible explanation for this effect is related to enzymatic inactivation at deep-freezing temperatures; this may have resulted in a decrease in the availability of enzymes responsible for the breaking down of collagen molecules [16, 44]. Although statistical significance was not achieved upon Bonferroni adjustment when comparing the 0.2% yield stress of allograft irradiated at higher freezing temperature and those which were non-irradiated, and when comparing the toughness of allograft irradiated at higher and deep-freezing temperatures, the magnitude of difference in effect size was large. An effect size of 1.0 indicated that the mean 0.2% yield stress of allograft irradiated at deep-freezing temperatures and mean toughness of the non-irradiated allograft were both at

Table 1. Mean and median values of the biomechanical parameters in the three experimental groups

Irradiated in gel ice group vs radiated in dry ice group				
Parameter	**Irradiated in gel ice group**	**Irradiated in dry ice group**	**Mean or median difference (95% CI)**	**p value**
0.2% offset yield stress in MPa	73 ± 28	101 ± 28	28 (3-52)	0.02
Ultimate compressive stress in MPa	84 ± 27	119 ± 31	35 (9-60)	0.005
Young modulus in GPa	2.80 (2.17)	3.51 (2.6)	0.71 (0.52 to 1.00)	0.21
Toughness in kJ/m^3	3622 ± 1720	5854 ± 2900	2232 (70-4394)	0.009
Irradiated in gel ice group vs non-irradiated control group				
Parameter	**Irradiated in gel ice group**	**Non-irradiated control group**	**Mean or median difference (95% CI)**	**p value**
0.2% offset yield stress in MPa	73 ± 28	109 ± 38	36 (11-60)	0.002
Ultimate compressive stress in MPa	84 ± 27	128 ± 40	44 (17-69)	< 0.001
Young modulus in GPa	2.80 (2.17)	3.49 (2.03)	0.81 (0.54 to 1.03)	0.21
Toughness in kJ/m^3	3622 ± 1720	6231 ± 3410	2609 (447-4771)	0.02
Irradiated in dry ice group vs non-irradiated control group				
Parameter	**Irradiated in dry ice group**	**Non-irradiated control group**	**Mean or median difference (95% CI)**	**p value**
0.2% offset yield stress in MPa	101 ± 28	109 ± 38	8 (-17 to 32)	> 0.99
Ultimate compressive stress in MPa	119 ± 31	128 ± 40	9 (-17 to 34)	> 0.99
Young modulus in GPa	3.51 (2.6)	3.49 (2.03)	-0.02 (-0.24 to 0.2)	0.21
Toughness in kJ/m^3	5854 ± 2900	6231 ± 3410	377 (-1785 to 2539)	0.74

Data presented as the mean ± SD, except for Young modulus, which is expressed as median (interquartile range), difference of medians (95% CI); p value of < 0.0167 is considered significant; MPa = megapascal, GPa = Gigapascal, kJ/m^3 = kilojoules per cubic meter.

the 84th percentile of those irradiated at higher temperature.

The results of this experiment are also in line with those of studies conducted on cancellous bone allografts. By subjecting cancellous bones to compression loads, three studies [2, 3, 17] reported no difference in mechanical properties for compressive strength and the Young modulus between control groups and groups irradiated at various doses under deep-freezing temperature conditions, except for one [2] in which a dose of 60 kGy induced weakening.

Our results differed from studies which concluded that cortical bone allograft tissue irradiated between 25 kGy and 35 kGy is weaker than non-irradiated cortical bone tissue [1, 9, 19, 21], and from papers that specifically stated that temperature during irradiation has little to no effect on the mechanical properties of cortical bone allografts [19, 21]. Although direct comparisons are difficult because of different study methods, an unregulated deep-freezing temperature during irradiation may have been responsible for these previous findings. Consistent with this speculation, in the current study, we found samples in the irradiated in gel ice group which were irradiated at a higher temperature range of -40°C to 0°C had worse compressive mechanical properties than those in the irradiated in dry ice group, which were irradiated at a deep-freezing temperature maintained below -40°C.

Similar to other studies [3, 17-19, 21], we did not observe a difference in the Young modulus between the three treatment groups. This reinforces an important concept, which is that the temperature during irradiation does not alter the stiffness of cortical allografts that is determined by the Young modulus [19]. The Young modulus values of our study were lower compared with preexisting studies [4, 35, 50], and this may have been attributed to the method of measurement of strain employed, such as direct measurement of relative displacement of the compression platens through cross head movement. Conventionally, the use of a Linear Variable Differential Transformer or an extensometer is recommended for more accurate strain evaluation [23, 45, 50]. Although the former device and its

mounting apparatus were not available to us, we found that the sample dimensions were too minute to be evaluated with the available extensometers. However, as we sought to compare the relative rather than absolute values of those mechanical parameters between each experimental group, we have reason to believe the most straightforward method adopted here was adequate [46, 50].

Conclusion

Our findings suggest that well-regulated, deep-freezing temperatures below -40°C during gamma irradiation may be a promising approach to better retain the native mechanical properties of cortical bone allografts. Future work involving other mechanical testing regimens, including tension, viscoelastic properties, fracture toughness, fatigue, and fatigue crack propagation, are necessary to verify and strengthen these findings. Biochemical and microscopic evaluation after mechanical testing would also allow us to better understand the underlying mechanistic reasons associated with the presumed retention of mechanical properties. Before we may recommend routine gamma irradiation of cortical bone allograft below deep-freezing temperatures, a pathogen inactivation study of the process at these temperatures must also be conducted to validate sterility, as all of these features contribute to the clinical success of bone allograft.

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