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Reducing the radiation sterilization dose improves mechanical and biological quality while retaining sterility assurance levels of bone allografts.

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Abstract

Background: Bone allografts carry a risk of infection, so terminal sterilization by gamma irradiation at 25 kGy is recommended; but is deleterious to bone quality. Contemporary bone banking significantly reduces initial allograft bioburden, questioning the need to sterilize at 25 kGy.

Methods: We inoculated allograft bone with *S. epidermidis* and *B. pumilus*, then exposed them to gamma irradiation at 0, 5, 10, 15, 20 and 25 kGy. Mechanical and biological properties of allografts were also assessed. Our aim was to determine an optimal dose that achieves sterility assurance while minimizing deleterious effects on allograft tissue.

Results: 20-25 kGy eliminated both organisms at concentrations from 10^1-10^3, while 10-15 kGy sterilized bone samples to a bioburden concentration of 10^2. Irradiation did not generate pro-inflammatory bone surfaces, as evidenced by macrophage activation, nor did it affect attachment or proliferation of osteoblasts. At doses above 15 kGy, both the modulus of toughness of cortical bone and attachment and fusion of osteoclasts onto irradiated bone, declined significantly. There was no change in collagen cross-links, but a significant dose-response increase in denatured collagen.

Conclusions Our mechanical and cell biological data converge on 15 kGy as a threshold for radiation sterilization of bone allografts. At or below this dose, bone banks can produce allografts providing an acceptable sterility assurance level, at which allograft strength and biocompatibility are improved significantly.

Clinical Relevance: The application of radiation sterilization doses below 15 kGy will improve bone allograft mechanical performance and promote integration, while retaining sterility assurance levels. Improved quality of allograft bone will promote superior clinical outcomes.

Introduction

Allogeneic tissue transplantation has been used for over 50 years. Over the past two decades, around 10 million allografts were implanted worldwide [1]. While biomedical engineering searches for effective tissue replacements, allograft bone remains a necessary graft material in orthopaedic surgery. The demand for allografts continues to increase. In the US, there were about 1.5 million allografts implanted in 2006, five times more than were implanted in 1996 [2]. The use of any allograft material carries with it the risk of transferring disease from the donor to the recipient. Among millions of tissue implantations, the American Association of Tissue Banks (AATB) reported an infection rate of 0.014% [1], some of which were fatal. Most of these infections occurred in allografts that were not subjected to terminal sterilization [3].

Allograft contamination is eliminated by donor screening, aseptic techniques during retrieval, processing and storage of the tissue [4]. Standards also ensure provision of allografts with a sterility assurance level (SAL) of 10^{-6} [5, 6]. These standards require that donor screening includes socio-medical history and serology, and that aseptic techniques are used during procurement, processing and packaging. Above all, gamma irradiation with a minimum dose of 25 kGy, the "Standard Dose", was recommended for terminal sterilization of bone allografts to provide the prescribed SAL. At the Standard Dose, gamma irradiation sterilizes bone allografts but also weakens them mechanically and reduces their biological activity. For example, ultimate stress, strain, and toughness of bone allografts are significantly reduced after irradiation at 25 kGy or more [7-9]. Allograft irradiation at 25 kGy dramatically increases the rate of allograft fractures (39%) compared with non-irradiated allografts (18%) [10]. Irradiation at the Standard Dose also reduces the osteoinductivity [11] and osteoconductivity [12, 13] of allografts, causing significant delays in host incorporation of the allograft [14].

During the past decade, a revolution in quality control in tissue banking has followed from the implementation of standards set by professional organizations such as AATB, International Atomic Energy Agency (IAEA), European Association of Tissue Banks (EATB), and government regulators such as the U.S. Food and Drug Administration (FDA) and Australian Therapeutic Goods Administration (TGA). As a result, the bioburden of allografts (being the number of microorganisms presenting on/in allografts prior to irradiation) is extremely low, or even zero [15]. Yet, many such regulatory authorities require that allografts undergo secondary sterilization. Recently, several tissue banks have successfully established radiation sterilization doses (RSD) for tissue allografts that are lower than the Standard Dose [15-17], with the lowest of these being 8.3 kGy [16]. The aim of lowering the RSD is to retain the prescribed SAL and to preserve the mechanical and biological properties of bone allografts, thereby leading to improved clinical outcomes for bone transplantation.

We hypothesize that tissue properties can be retained, with the required SAL of 10^{-6}, at a radiation sterilization dose between 10 and 15 kGy. Therefore, using a known bioburden, we sought to determine the threshold dose of radiation at which the SAL could be achieved, and its effect on the mechanical properties, biocompatibility and collagen biochemistry at different gamma doses. The broad objective of this work is to recommend a radiation sterilization dose providing the required SAL, while optimizing the biological and mechanical performance of the allograft.

Materials and methods

Bone sample preparation

With consent from the donor, or the donors' next of kin, 16 femora from 8 donors were provided by the Queensland Bone Bank (QBB). The Human Ethics Committee of the Queensland Health Forensic and Scientific Services approved the project.

Femora were processed according to QBB standards in the clean processing room and stored at -75°C. Each femur provided a femoral head, four cortical (diaphyseal) portions (l = 4 cm) and two cancellous blocks. It's important to note that advances in tissue banking have led to significant reductions in the bioburden of processed bone allografts (ie prior to secondary sterilisation). For example, a 12-month survey of QBB processing, demonstrated that bioburden of all types of bone allografts manufactured at QBB were zero (H. Nguyen, unpublished data). Therefore, to establish valid recovery efficiencies, specimens were inoculated with bacterial species as described below.

Soft tissue and cartilage was removed from femoral heads, they were cut into small chips (0.3 g), washed with warm sterile saline, and soaked in 6% hydrogen peroxide and 70% ethanol for 10 min. One femoral head provided 36 bone chips for bacterial inoculation experiments. Bone chips were inoculated with either *Staphylococcus epidermidis* or *Bacillus pumilus* at 10^1, 10^2 and 10^3 organisms/chip and allocated to 6 groups (Table 1).

For 3-point bending tests, 3 cortical portions were sectioned into 6 beams (40 × 4 × 2 mm) per portion using a low speed saw (Leco saw, USA). There was no significant difference in mechanical properties among cortical bone beams from the same portion (data not shown). Therefore, 6 cortical bone beams from the same portion were allocated into 6 irradiation groups. Eight donors provided 48 cortical portions, giving 288 cortical beams (Table 2). The remaining cortical portion was sectioned longitudinally into cortical bone rods (40 × 5 × 5 mm). These were

either sliced into 100-µm-thick sections for tissue culture or ground for biochemical analysis (Table 2).

Two cancellous bone blocks (60 × 20 × 20 mm) from femoral condyles were cut to obtain 12 cancellous bone cubes (15 × 10 × 10 mm). Six specimens from each block were randomly allocated to 6 groups for compression testing (Table 2).

Gamma irradiation (cobalt-60)

Specimens in gamma treatment groups were irradiated, frozen, at 5, 10, 15, 20, and 25 kGy at the Australian Nuclear Science and Technology Organisation, Lucas Heights, Australia. Control specimens remained in a freezer. Dose-mapping verified that the delivered doses were 5.1 (4.7 – 5.5) kGy, 10.7 (9.9 – 11.5) kGy, 16.7 (15.5 – 17.9) kGy, 22.7 (21.1 – 24.3) kGy, and 26.6 (24.8 – 28.5) kGy.

Experimental methods

Sterility testing: The method was adopted from International Standard, ISO 11737-1.2006 [18], the Australian Therapeutic Goods Administration (TGA) guideline for sterility testing of medical products [19] and previous publications [20, 21].

B. pumilus (ATCC 27142 - SGM biotech, USA) and *S. epidermidis* (ATCC 12228 - Australian Collection of Microorganisms - AMC) were supplied in stock suspensions. Working suspensions of 10^1, 10^2, and 10^3 organisms/0.1 mL were prepared according to the provider's instructions.

Bone chips were individually placed in 2 mL tubes according to organism type and population, and gamma dose; whereupon 0.1 mL of the bacterial suspension was added to the bone. Bone

chips without bacterial inoculation, or irradiation, served as negative controls. The population in each working suspension was verified by direct agar plate inoculation (Table 3).

After irradiation, sample tubes were filled with sterile saline, vortex mixed and the solution was filtered through Micropressure system membranes (Millipore, USA). The filter membranes were removed and placed onto either tryptic soy agar plates for *B. pumilus* or Corynebacterium agar plates for *S. epidermidis*. Plates were incubated at 30°C (*B. pumilus)* or 37°C (*S. epidermidis)* for 7 d. For the purpose of the sterility test, plates were observed and read as growth (non-sterile) or no-growth (sterile).

The TGA guideline for sterility testing [19] recommends that the efficiency of a removal method must be more than 50%. Efficiency of membrane filtration in our testing was 83% for *S. epidermidis* and 70% for *B. pumilus* (data not shown).

Three-point bending: Irradiated cortical bone specimens were thawed at room temperature for 30 min. Bones were tested using an Instron 8722 servo-hydraulic materials testing machine, according to previously published methods [22]. Briefly, specimens were placed on two rigid brass supports 24 mm apart and tested in three-point loading, with the actuator and its attached load-cell applying load to the mid-span. Actuator speed was 1 mm/s. Load-displacement data were acquired using Wavemaker software (Instron, UK). The yield point was defined using a 0.2 % offset.

Structural properties, determined from load-displacement curves, were used to calculate material properties as follows [23]:

$\delta = F(3l/2wt^3)$	Where δ: stress ε: strain	S: stiffness (F/d)
$\varepsilon = d(6t/l^2)$	E: elastic modulus	U: work to failure
$E = S(l^3/4wt^3)$	u: toughness modulus	l: the span of the loader (24 mm)
$u = U(9/lwt)$	F: applied force	w: specimen width (4 mm)
	d: loader displacement	t: specimen thickness (2 mm)

Compressive testing: Frozen irradiated cancellous bone cubes were thawed at room temperature for 30 min. Each specimen was compressed in the longitudinal direction between two platens at 0.5 mm/s. The lower platen pivoted on a ball bearing to accommodate conformational changes during compression. The test was stopped when the sample was compressed to 25% of its original height. Structural properties taken from the load-displacement graph were used to calculate the material properties of the cancellous bone [24]. Mechanical data were subsequently normalised for bone volume fraction (BV/TV), using data from a 70 μm-thick histological section from each specimen.

$\delta = (F/CSA)/BVF$	Where δ: stress	BVF: bone volume fraction
$E_n = (S*(h/CSA))/BVF$	E_n: normalized elastic modulus	S: stiffness (F/d)
$u = (U/(h/CSA))/BVF$	u: toughness modulus	U: Work to failure
	F: applied force (N)	h: specimen height (m)
	CSA: cross sectional area (m)	

Tissue culture experiments

Fluorescence Activated Cell Sorter/Flow Cytometry (FACS) analysis: RAW264.7 murine macrophage cells stably transfected with a construct containing the E-selectin (ELAM) promoter, to drive expression of enhanced green fluorescent protein (eGFP), can be used to assay pro-inflammatory signaling [25, 26]. To determine if irradiation produced pro-inflammatory mediators, RAW264.7/ELAM-eGFP cells were incubated on control and irradiated cortical bone slices, and eGFP expression was analyzed by FACS. Irradiated bone slices were placed in 24 well tissue culture plates and RAW264.7/ELAM-eGFP macrophages were seeded on the bone slices at 3×10^5 cells/slice. Cells treated with 10 ng/mL of *S. minnesota* LPS (Sigma) served as a positive control and cells cultured without bone served as a negative control. The cultures were incubated for 24 h at 37°C. The cells were harvested and re-suspended in 400 µL of PBS.

Activated cells were detected using a FACS-Calibur flow cytometer (Becton Dickinson, USA). Data analysis was performed by analyzing 10,000 events for each assay using CELLQuest software package (Becton Dickinson, USA). Fluorescence intensity was divided into two regions, M1 (background level of cellular autofluorescence) and M2 region (the logarithmic increment of fluorescence intensity). All events with fluorescence intensity greater than the M1 region were accepted as cellular eGFP fluorescence events.

Enzyme-linked immunoabsorbent assay (ELISA): Following FACS analysis, the supernatant was harvested for ELISA detection of Tumor Necrosis Factor (TNF) secretion. TNF ELISA was performed using a mouse TNF ELISA kit (BD, Biosciences cat 560478), following the manufacturer's instructions.

Osteoblast attachment assay[27]: Sterile bone slices were placed in the corresponding wells of 96 well plates. One mL of cell suspension containing 2×10^4 cells/mL of MC3T3-E1 cells was added into each well. The plates were incubated at 37°C for 2 h, whereupon the culture medium was removed from wells and bone slices were washed three times with PBS. A 50 µL aliquot of MTT reagent (1 ng/mL; Promega, USA) and a 50 µL aliquot of complete medium were added to each well. Plates were incubated for an additional 45 min at 37°C, media was removed and replaced by 100 µL of isopropanol and the plates were incubated for 10 min with gentle agitation to lyse the cells. Finally, the solution was transferred to new plates and read at 570 nm. The absorbance values from non-washed correspondent wells served as control.

Osteoblast proliferation assay[27]: The tissue-culture plastic was pre-coated with 30 µL/cm^2 12% poly(2-hydroxyethyl methacrylate) (PHEMA) in 95% ethanol (Sigma) and allowed to harden overnight at 37°C. On the following day, the bone slices were placed into wells and the MC3T3-E1 cells were seeded at a density of 2×10^3 cells/well. The wells were top up with the completed medium to 200 µL and incubated for either 2 or 4 d at 37°C.

At the designated time point, the medium was removed and bone slices were washed with PBS to removed residual medium and non-attached cells. A 50 µL aliquot of MTS reagent (1 ng/mL) (Promega, USA) was added into each well and incubated for 4 h whereupon the solution was transferred to a new plates and the absorbance at 490 nm was measured using a plate reader.

Osteoclast assays: Osteoclast-like cells (Oc) were induced to differentiate *in vitro* by culturing bone marrow cells isolated from BALB/c mice on control or irradiated bone slices in the presence of the cytokines, M-CSF and soluble RANKL. Osteoclast formation and fusion were assayed by measuring the number of Oc, the number of their nuclei, and the Oc cell area using light

microscopy (Olympus BX60). Bone marrow cells were flushed with tissue culture medium from femurs and tibias of 8 weeks old BALB/c mice and the suspension was further diluted with complete DMEM medium to produce the desired bone marrow cell suspension of 2.5×10^4 cells/mL.

Irradiated bone slices in wells were seeded with 100 µL of bone marrow cell suspension and 100 µL of medium was added and incubated at 37°C for 7 d. At the end of the incubation time, culture medium was removed and cells attached to the slices were fixed and stained for tartrate-resistant acid phosphatase (TRAP). Bone slices were mounted onto glass slides and the number of multinucleated TRAP positive cells (three or more nuclei – osteoclast-like cells, Oc) and the number of nuclei in these Oc per slide was counted under light microscopy. Five pictures were randomly taken from each slice. ImageJ (NIH-image software for PC) software was used to measure the area of Oc on these images.

Collagen biochemistry

The collagen matrix of bone largely determines its post-yield properties. Therefore we undertook biochemical analyses of collagen and its cross-links. Bone samples were cut in small pieces and then defatted with methanol chloroform (1:3). Methods for measuring collagen enzymatic (pyridinoline – PYD, deoxypyridinoline – DPD), and non-enzymatic (pentosidine – PEN) and immature (dihydroxynorleucine – DHLNL and hydroxynorleucine - HLNL) crosslinks were then assayed using methods previously published[28].

Collagen degradation was determined using the alpha chymotrypsin (αCT) method [29]. Bone samples were powdered, defatted and demineralised by EDTA. Demineralised bone samples were digested overnight at 37°C with 1 mg/mL of αCT. After centrifugation, supernatants and

pellets containing αCT-solubilized fragments and intact collagen molecules respectively were analysed for hydroxyproline content. The amount of denaturated collagen was expressed as the percentage of total collagen.

Statistical analysis

Non-parametric tests (Kruskal-Wallis Test) assessed data in bacterial experiments. The bivariate correlation – one-tailed (Pearson's correlation coefficient) assessed the relationship between gamma dose and sterility of samples inoculated with different levels of organism populations and irradiated at different doses, considered as significant if $P < 0.05$. The results were presented as sterile (no-growth) or non-sterile (growth).

One-way ANOVA analyzed differences among groups in mechanical, biocompatibility and biochemical experiments. When significant, Tukey's Honest Significance Test defined differences between groups, significant if $P < 0.05$. Results were presented as mean +/- SE.

The relationships between gamma dose, crosslinks, and denatured collagen percentage and allograft properties *e.g.,* toughness and number of OCLs formed per bone slice were analyzed by regression models. The significance was accepted at $P < 0.05$.

Results

Sterility status

Bone samples inoculated at 10^3 organisms had a very high bioburden and only samples irradiated at 20 – 25 kGy were negative for growth. Growth of both *S. epidermidis* and *B. pumilus* was observed for samples irradiated up to 15 kGy (Table 4 and 5), and their response was dose-

dependent ($r^2 = 0.56$, $P < 0.0001$). Samples inoculated with either organism at concentrations of 10^1 or even 10^2, were sterilized with a dose as low as 10 kGy (Table 4 and 5).

Three-point bending

ANOVA confirmed significant reductions in both yield stress and strain (Table 6), the point at which damage is initiated. These reductions were significant for gamma doses of 20 and 25 kGy ($P < 0.05$, Tukey's Test), but there was no dose-response reduction in elastic modulus.

In contrast, stress at failure was not significantly altered, but a dose-related reduction in failure strain was observed ($P < 0.001$). Consequently, the toughness modulus was significantly reduced ($P < 0.001$; Table 6). Failure strain and toughness modulus declined significantly at 10 kGy (11 and 20%, respectively), and these reductions were much greater at 20 and 25 kGy. No significant changes in cancellous bone material properties were observed (Table 7).

Pro-inflammatory analysis

Incubation with non-irradiated bone induced an activation of eGFP expression after 24 h, but there was no significant dose-related increase in eGFP expression between control and irradiated groups ($P > 0.05$). Data from the ELISA verified that TNF production by cells was not increased by irradiation, and corresponded to the pattern of eGFP expression (Fig. 1A).

Bone cell assays

There was growth of MC3T3E1 cells over time (Fig. 2), but there was no significant effect on absorbance indices of attachment or proliferation (Fig. 1B) of osteoblastic cells seeded on to irradiated bone slices. After incubation for 7 d on irradiated bone slices, the number of TRAP

positive Oc, the number of their nuclei and Oc cell area significantly decreased (Fig. 1C-F). These changes were significant for gamma doses greater than 15 kGy ($P < 0.01$). Linear regression analysis showed a strong relationship between gamma dose and impaired Oc formation ($r^2 = 0.62$ and $P < 0.001$).

Collagen Biochemistry

Although trends were observed for reductions in collagen crosslinks, these changes were not statistically significant ($P > 0.05$). Analysis of the concentration of degraded collagen revealed a significant dose-dependent increase in denatured collagen ($P < 0.001$). This degradation became significant at a gamma dose of 15 kGy with twice the amount of degradation at 25 kGy (Table 8).

Relationships among collagen, toughness and osteoclast formation

There was a significant, but small, correlation between gamma dose and bone toughness ($r^2 = 0.20$, $P < 0.05$). Osteoclast (Oc) numbers were significantly reduced on bone slices irradiated at higher gamma doses (Fig. 2), and the concentration of denatured collagen was significantly increased ($r^2 = 0.7$, $P < 0.0001$, Fig. 2). Further analysis revealed that PEN predicted the changes in bone toughness ($r^2 = 0.62$ at $P < 0.0001$) during gamma irradiation, while denatured collagen predicted decreased Oc formation on bone slices ($r^2 = 0.62$, $P < 0.0001$).

Combining analyses for toughness, osteoclast formation and denatured collagen (Tables 6, 8 and Fig. 2) demonstrated that these variables converge at a threshold dose around 15 kGy, below which the tissue properties were maintained and above which bone quality was significantly reduced (Fig. 3).

Discussion

We have demonstrated that a reduction in radiation sterilization dose is justified because the quality of the bone tissue can be maintained, while retaining the required SAL. We also observed that collagen degradation plays a large role in the changes in mechanical properties, and that altered osteoclast viability could explain poor clinical outcomes in remodeling and the incorporation of the allograft into the host tissue. In the dose range encompassing 25 kGy, loss of bone toughness was related to denaturation of collagen molecules. It is also significant, that 15 kGy emerged as a threshold dose, below which the mechanical properties, collagen degradation and osteoclast viability were maintained at values similar to control allografts. Clinically, the improvement in toughness modulus reflects a greater capacity of the material to absorb energy from daily loading, increasing the resistance to crack propagation, and allograft failure[30]. Cancellous bone appears to be more resistant to irradiation so that the standard gamma dose does not affect its biomechanical properties, consistent with the observations of others[31].

To determine a threshold gamma dose that provides the necessary SAL, *S. epidermidis* was selected as the most common contaminant on bone allografts [32-37], and *B. pumilus* was selected as a highly gamma-irradiation resistant organism (D_{10} value of 1.8 kGy, the dose required to kill 90% of presenting organisms) [20, 21, 37, 38]. Sterility testing revealed that all inoculated samples irradiated at 20 and 25 kGy were negative for growth, at any concentration. Irradiation at 10 and 15 kGy, provided sterility for samples inoculated at 10^1 and 10^2 populations (Tables 4 and 5). These represent bioburdens that are higher than those now achieved in bone bank processing [15]. Data presented here, and our recent validations studies[15, 17], suggest that a RSD of 10 kGy is sufficient to maintain the biological and mechanical properties of allografts, while retaining the required SAL.

For allograft incorporation to occur, bone remodeling must replace the allograft with new bone tissue over time. Resorption is the first stage of remodeling and is initiated when osteoclasts (Oc) are activated. We observed a dose-dependent deterioration in osteoclastogenesis for Oc cultured on irradiated bone slices. Critically, Oc formation and fusion were less than 40% of control groups when cultured on bone irradiated at 25 kGy, yet the number of Oc attached to bone slices was about 80% when bone was irradiated at 15 kGy (Fig. 3). In a clinical setting, poorly formed Oc affect bone resorption and consequently the incorporation of the allograft[12]. Sterilizing allograft bone at doses below 15 kGy, will therefore maintain its mechanical properties and the potential of the bone tissue to support remodeling, and to promote incorporation. Importantly, the level of eGFP expression was not increased when macrophages were incubated on irradiated bone slices. This indicates that products generated in the bone matrix by irradiation do not stimulate pro-inflammatory activity that could lead to osteolysis.

Following resorption, osteoblasts attach to the bone surface, proliferate and subsequently differentiate. During this process they secrete factors such as receptor activator of NF-κB ligand and osteoprotegerin that regulate osteoclastogenesis. Irradiation did not influence either the attachment of osteoblasts onto irradiated bone slices or their proliferation on these slices. These data support earlier observations [38], in which attachment and proliferation of bone marrow stromal cells was no different on cortical bone irradiated at 30 kGy compared to fresh frozen cortical bone slices.

Gamma irradiation did not significantly alter collagenous crosslinks; but they are disrupted if irradiation dose approaches 60 kGy [39]. In contrast, we observed greater collagen degradation at 15 kGy, with a dose-response increase in denatured collagen to 25 kGy ($r^2 = 0.7$; $P < 0.0001$).

Similar observations have been made for a 41% increase in denatured collagen in bone irradiated at 30.2 kGy [39, 40]. Although alterations in mechanical properties were related to collagen denaturation ($r^2 = 0.11$ at $P < 0.05$), regression analysis showed that mechanical properties were more strongly dependent on the change in crosslinks, especially the non-enzymatic crosslink pentosidine ($r^2 = 0.38$ at $P < 0.001$). Conversely, the reduction in osteoclast formation was more strongly dependent on the amount of denatured collagen ($r^2 = 0.45$ at $P < 0.001$), and did not correlate significantly with crosslink content. In short, crosslink content measurements were good predictors for mechanical alterations while denatured collagen was a good predictor for the osteoconductivity of bone allografts.

Conclusions

Our data verify that doses of 20-25 kGy eliminate high levels of a known bioburden inoculated into bone samples, but that lower doses of gamma radiation at 10-15 kGy also eliminate bioburden within a range associated with processed allografts. Irradiation did not generate proinflammatory bone surfaces, evidenced by macrophage activation, nor did it affect attachment or proliferation of osteoblasts. At gamma doses $\geq$ 10 kGy, the toughness of cortical bone declined, while the attachment and fusion of osteoclasts onto irradiated bone declined significantly at doses > 15 kGy. We observed no significant change in crosslink content, but a dose-response increase in denatured collagen in irradiated bones, underlying the degradation in mechanical competence and osteoclast biology.

The significant advance for bone banking is that mechanical and cell biological data converge on 15 kGy as a threshold for radiation sterilization of bone allografts. At, or below, this dose bone

banks can produce allografts that provide an acceptable sterility assurance level, and at which allograft strength and biocompatibility are improved significantly.

References

[1] Vangsness CT, Dellamaggiora RD. Current Safety Sterilization and Tissue Banking Issues for Soft Tissue Allografts. Clin Sports Med 2009;28: 183-187.

[2] McAllister DR, Joyce MJ, Mann BJ, Vangsness CT, Jr. Allograft Update: The Current Status of Tissue Regulation, Procurement, Processing, and Sterilization. Am J Sports Med 2007;35: 2148-2158.

[3] Harner CD, Lo MY. Future of Allografts in Sports Medicine. Clin Sports Med 2009;28: 327-340.

[4] Nguyen H, Morgan DA, Forwood MR. Sterilization of allograft bone: is 25 kGy the gold standard for gamma irradiation? Cell Tissue Bank 2007;8: 81-91.

[5] AATB. Standards for Tissue Banking. In. 10th ed. Maryland: Am Ass. of Tissue Banking; 2002. p. 142.

[6] International Atomic Energy Agency (IAEA). Code of Practice for the Radiation Sterilization of Tissue Allografts: Requirements for Validation and Routine Control. In: IAEA, editor. The IAEA INT/6/052 Programme in Radiation and Tissue Banking; 2005. p. 47.

[7] Currey JD, Foreman J, Laketic I, Mitchell J, Pegg DE, Reilly GC. Effects of ionizing radiation on the mechanical properties of human bone. J. Orthop. Res. 1997;15: 111-117.

[8] Hamer AJ, Strachan JR, Black MM, Ibbotson CJ, Stockley I, Elson RA. Biomechanical properties of cortical allograft bone using a new method of bone strength measurement - A comparison of fresh, fresh-frozen and irradiated bone. J. Bone Joint Surg.-Br. Vol. 1996;78: 363-368.

[9] Akkus O, Belaney RM, Das P. Free radical scavenging alleviates the biomechanical impairment of gamma radiation sterilized bone tissue. J. Orthop. Res. 2005;23: 838 - 845.

[10] Lietman SA, Tomford WW, Gebhardt MC, Springfield DS, Mankin HJ. Complications of irradiated allografts in orthopaedic tumor surgery. Clin. Orthop. Rel. Res. 2000: 214-217.

[11] Buring K, Urist RM. Effects of Ionizing Radiation on the Bone Induction Principle in the Matrix of Bone Implants. Clin. Orthop. Rel. Res. 1967;55: 225-234.

[12] Kluger R, Bouhon W, Freudenberger H, Kroner A, Engel A, Hoffmann O. Removal of the surface layers of human cortical bone allografts restores in vitro osteoclast function reduced by processing and frozen storage. Bone 2003;32: 291-296.

[13] Munting E, Wilmart JF, Wijne A, Hennebert P, Delloye C. Effect of Sterilization on Osteoinduction - Comparison of 5 Methods in Demineralized Rat Bone. Acta Orthop. Scand. 1988;59: 34-38.

[14] Voggenreiter G, Ascherl R, Blumel G, SchmitNeuerburg KP. Extracorporeal irradiation and incorporation of bone grafts - Autogeneic cortical grafts studied in rats. Acta Orthop. Scand. 1996;67: 583-588.

[15] Nguyen H, Morgan DAF, Sly LI, Benkovich M, Cull S, Forwood MR. Validation of 15 kGy as a radiation sterilisation dose for bone allografts manufactured at the Queensland Bone Bank: application of the VDmax 15 method. Cell and Tissue Banking 2008;9: 139-147.

[16] Moore M, Linthurst AJ, Gaskin B, Wolfinbarger L. Adaptation of ANSI/AAMI/ISO 11137 method 2B Sterilisation Validation for Medical Devices to Tissue Banking. In: American Association of Tissue Banks - 28th Annual Meeting. Chicago, Illinois, USA; 2004.

[17] Nguyen H, Morgan DA, Forwood MR. Validation of 11 kGy as a radiation sterilization dose for frozen bone allografts. J Arthroplasty 2011;26: 303-308.

[18] ISO 11737-1. Sterilization of medical devices - Microbiological methods. In: Part 1: Determination of a population of microorganisms on products: International Standard Organisation; 2006, p. 35.

[19] TGA. TGA guideline for sterility testing of therapeutic goods. In: Australian Government - Department of Health and Aging - Therapeutic Goods Administration, editor. Canberra; 2006. p. 33.

[20] Burd T, Conroy BP, Meyer SC, Allen WC. The effects of chlorhexidine irrigation solution on contaminated bone-tendon allografts. Am. J. Sports Med. 2000;28: 241-244.

[21] Soyer J, Rouil M, Castel O. The effect of 10% povidone-iodine solution on contaminated bone allografts. J. Hosp. Infect. 2002;50: 183-187.

[22] Bennell KL, Khan KM, Warmington S, Forwood MR, Coleman BD, Bennett MB, Wark JD. Age does not influence the bone response to treadmill exercise in female rats. Med. Sci. Sports Exerc. 2002;34: 1958-1965.

[23] Turner CH, Burr D. Experimental techniques for Bone Mechanics. In: Cowin SC, editor. Bone Mechanics Handbook. Boca Raton: CRC; 2001, p. 7-1 - 7-35.

[24] Allen RM, Iwata K, Sato M, Burr D. Raloxifence ENhances Vertebral Mechanical Properties Independent of Bone Density. Bone 2006;39: 1130 - 1135.

[25] Hume DA, Underhill DM, Sweet MJ, Ozinsky AO, Liew FY, Aderem A. Macrophages exposed continuously to lipopolysaccharide and other agonists that act via toll-like receptors exhibit a sustained and additive activation state. BMC Immunol 2001;2: 11.

[26] Wu ACK, Grondahl L, Jack KS, Foo MX, Trau M, Hume DA, Cassady AI. Reduction of the in vitro pro-inflammatory response by macrophages to poly(3-hydroxybutyrate-co-3-hydroxyvalerate). Biomaterials 2006;27: 4715-4725.

[27] Cool SM, Kenny B, Wu A, Nurcombe V, Trau M, Cassady AI, Grondahl L. Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) composite biomaterials for bone tissue regeneration: In vitro performance assessed by osteoblast proliferation, osteoclast adhesion and resorption, and macrophage proinflammatory response. Journal of Biomedical Materials Research, Part A 2007;82A: 599-610.

[28] Viguet-Carrin S, Follet H, Gineyts E, Roux JP, Munoz F, Chapurlat R, Delmas PD, Bouxsein ML. Association between collagen cross-links and trabecular microarchitecture properties of human vertebral bone. Bone 2010;46: 342-347.

[29] Bank RA, Krikken M, Beekman B, Stoop R, Maroudas A, Lafebers FPJG, Te Koppele JM. A simplified measurement of degraded collagen in tissues: Application in healthy, fibrillated and osteoarthritic cartilage. Matrix Biology 1997;16: 233-243.

[30] Michell JE, Stawarz MA, Kayacan R, Rimnac MC. The Effects of Gamma Radiation Sterilisation on the Fatigue Crack Propagation Resistance of Human Cortical Bone. Journal of Bone and Joint Surgery 2004;86: 2648 - 2657.

[31] Anderson MJ, Keyak JH, Skinner HB. Compressive Mechanical-Properties of Human Cancellous Bone after Gamma-Irradiation. J. Bone Joint Surg.-Am. Vol. 1992;74A: 747-752.

[32] Deijkers RLM, Bloem RM, Petit PLC, Brand R, Vehmeyer SBW, Veen MR. Contamination of bone allografts - Analysis of incidence and predisposing factors. J. Bone Joint Surg.-Br. Vol. 1997;79: 161-166.

[33] Journeaux SF, Johnson N, Bryce SL, Friedman SJ, Sommerville SMM, Morgan DAF. Bacterial contamination rates during bone allograft retrieval. J. Arthroplast. 1999;14: 677-681.

[34] Liu JW, Chao LH, Su LH, Wang JW, Wang CJ. Experience with a bone bank operation and allograft bone infection in recipients at a medical centre in southern Taiwan. J. Hosp. Infect. 2002;50: 293-297.

[35] Lord CF, Gebhardt MC, Tomford WW, Mankin HJ. Infection in Bone Allografts - Incidence, Nature and Treatment. J. Bone Joint Surg.-Am. Vol. 1988;70: 369-376.

[36] Sommerville SMM, Johnson N, Bryce SL, Journeaux SF, MORGAN DAF. Contamination of banked femoral head allograft: Incidence, bacteriology and donor follow up. Aust. N. Z. J. Surg. 2000;70: 480-484.

[37] Vehmeyer S, Wolkenfelt J, Deijkers R, Petit P, Brand R, Bloem R. Bacterial contamination in postmortem bone donors. Acta Orthop. Scand. 2002;73: 678-683.

[38] Hofmann A, Konrad L, Hessmann MH, Kuchle R, Korner J, Rompe JD, Rommens PM. The influence of bone allograft processing on osteoblast attachment and function. J. Orthop. Res. 2005;23: 846-854.

[39] Hamer A, Colwell A, Eastell R. Biomechanical and biochemical changes in cortical allograft bone after gamma irradiation. Bone 1996;19: 696-696.

[40] Hamer AJ, Stockley I, Elson RA. Changes in allograft bone irradiated at different temperatures. Journal of Bone and Joint Surgery. British Volume 1999;81: 342-344.

Figure Legends

Figure 1. (A) Pro-inflammatory assays of RAW264-7/ELAM-eGFP cells show no dose-related activation of macrophages (fluorescence of eGFP by FACS after incubation with irradiated bone slices). There is also no corresponding increase in TNF levels in the supernatant (absorbance values for ELISA x 100). (B) Neither attachment nor proliferation of osteoblast-like cells, MC3T3-E1, was altered when cultured on to irradiated bone slices [absorbance values represent the intensity of the MTT or MTS reagent from the cells incubated with irradiated bone at 2 hrs (attachment assay, ^{a} = absorbance at 570 nm), 2 days and 4 days (proliferation assay, ^{a} = absorbance at 490 nm)]. (C-F) Formation and fusion features of TRAP+ Oc cultured on bone slices irradiated from 0 to 25 kGy. (C) Average number of Oc per slice. (D) Average number of nuclei per Oc. (E) Average Oc size. (F) Relative Oc area (Oc.Ar/B.Ar, %). * = $P < 0.01$ compared to 0-15 kGy.

Figure 2: The relationship between denatured collagen (% total collagen) ($y = 0.78 * \text{Dose} + 15.70$; $r^2 = 0.7$, $P < 0.0001$), osteoclast numbers (Oc.N/Slice) ($y = 0.58 * \text{Dose} + 231.90$; $r^2 = 0.62$, $P < 0.001$) and dose of gamma irradiation (kGy).

Figure 3: Percentage change in values for denatured collagen, bone toughness and Oc number (all in %) according to increasing gamma-irradiation.

Table(s)

Table 1: Bone samples taken from 16 femoral heads for sterility testing of inoculated and irradiated bone samples

Dose (kGy)		0	5	10	15	20	25
Inoculated species	Population						
S. epidermidis	10^1	16	16	16	16	16	16
	10^2	16	16	16	16	16	16
	10^3	16	16	16	16	16	16
B. pumilus	10^1	16	16	16	16	16	16
	10^2	16	16	16	16	16	16
	10^3	16	16	16	16	16	16

Table 2

Table 2: Bone samples taken from eight donors for mechanical, biocompatibility and biochemical experiments

Dose (kGy)		0	5	10	15	20	25
Experiments							
Mechanical	Three-point bending	48	48	48	48	48	48
	Compression	32	32	32	32	32	32
Tissue culture	Macrophage-activation	8	8	8	8	8	8
	Osteoblast proliferation	8	8	8	8	8	8
	Osteoblast attachment	8	8	8	8	8	8
	Osteoclast formation	8	8	8	8	8	8
Biochemical	Crosslink analysis	8	8	8	8	8	8
	Denatured collagen	8	8	8	8	8	8

Table 3

Table 3: The organism population (per 0.1 mL) of suspensions inoculated on to bone chips

Species	10^3 suspension	10^2 suspension	10^1 suspension
S. epidermidis	2.0×10^3	3.7×10^2	4.7×10^1
B. pumilus	0.6×10^3	5.7×10^2	0.8×10^1

Table 4

Table 4: Outcome of gamma-irradiation sterility test for growth of *S. epidermidis.*

Dose (kGy)	N	Number of Positive Samples		
		10^3 inoculation	10^2 inoculation	10^1 inoculation
0	16	16	16	16
5	16	11	5	4
10	16	2	0	0
15	16	6	0	0
20	16	0	0	0
25	16	0	0	0
P		$< 10^{-6}$	$< 10^{-6}$	$< 10^{-6}$

Table 5

Table 5: Outcome of gamma-irradiation sterility test for growth of *B. pumilus*.

Dose (kGy)	N	Number of Positive Samples		
		10^3 inoculation	10^2 inoculation	10^1 inoculation
0	16	16	16	16
5	16	16	5	0
10	16	0	0	0
15	16	9	0	0
20	16	0	0	0
25	16	0	0	0
P		$< 10^{-6}$	$< 10^{-6}$	$< 10^{-6}$

Table 6

Table 6: Material properties of gamma-irradiated cortical bones (n = 48)

Dose (kGy)	δ_y (MPa)	ε_y (%)	E (GPa)	δ_f (MPa)	ε_f (%)	u (MJ/m^3)
0	93 ± 1.17	0.87 ± 0.01	10.76 ± 0.23	166 ± 3.22	2.74 ± 0.08	3.11 ± 0.14
5	89 ± 0.87	0.84 ± 0.01	10.71 ± 0.18	158 ± 2.83	2.72 ± 0.07	2.97 ± 0.13
10	89 ± 0.78	0.83 ± 0.01	10.85 ± 0.20	157 ± 2.55	2.45 ± 0.06 [a]	2.57 ± 0.09 [a]
15	91 ± 1.17	0.82 ± 0.01	11.24 ± 0.19	162 ± 3.18	2.43 ± 0.07 [a]	2.72 ± 0.13 [a]
20	88 ± 0.89[a]	0.80 ± 0.01[a]	10.92 ± 0.20	156 ± 3.30	2.26 ± 0.06 [a]	2.34 ± 0.10 [a]
25	87 ± 1.89[a]	0.80 ± 0.02[a]	10.88 ± 0.25	155 ± 2.97	2.24 ± 0.05[a]	2.31 ± 0.09 [a]
P[1]	< 0.01	< 0.01	NS	NS	< 0.001	< 0.001

Abbreviations: δ – Stress, ε – strain, y – yield, f – failure, E – Elastic modulus, u – toughness modulus. 1. Analysis of variance; a = P < 0.05 compared with 0-10 kGy, Tukey's test.

Table 7

Table 7: Material properties of gamma-irradiated cancellous bone (n = 32)

Dose (kGy)	δ_y (MPa)	δ_f (MPa)	E^n (MPa)	u (MJ/m^3)
0	15 ± 1.13	18 ± 1.36	467 ± 37	604 ± 39
5	15 ± 0.95	21 ± 1.49	502 ± 32	511 ± 48
10	13 ± 1.04	17 ± 1.32	465 ± 33	524 ± 55
15	15 ± 1.27	18 ± 1.52	465 ± 33	527 ± 45
20	14 ± 0.95	19 ± 1.20	489 ± 29	525 ± 53
25	15 ± 1.02	18 ± 1.15	443 ± 31	536 ± 35
P	> 0.05			

Table 8

Table 8: Crosslink contents (mmol/mol collagen) and denatured collagen (% of total collagen) for gamma-irradiated bone

Dose (kGy)	N	PEN	DPD	PYD	HLNL	DHLNL	Denatured Collagen
0	8	6.9 ± 1.4	158 ± 13	324 ± 18	252 ± 29	607 ± 83	16.88 ± 0.76
5	8	7.3 ± 1.4	150 ± 7	320 ± 12	236 ± 15	563 ± 55	19.37 ± 0.83
10	8	7.3 ± 1.4	136 ± 8	310 ± 18	253 ± 27	587 ± 78	21.15 ± 1.11
15	8	6.5 ± 1.1	137 ± 8	308 ± 12	275 ± 26	612 ± 70	27.48 ± 1.83[a]
20	8	6.3 ± 1.3	146 ± 8	310 ± 9	291 ± 25	630 ± 65	33.40 ± 2.44 [a]
25	8	6.5 ± 1.4	134 ± 13	288 ± 16	295 ± 26	649 ± 71	34.51 ± 1.63 [a]
P		> 0.05					< 0.001

a = $P < 0.05$ compared to 0-10 kGy (Tukey's test).

Figure 1

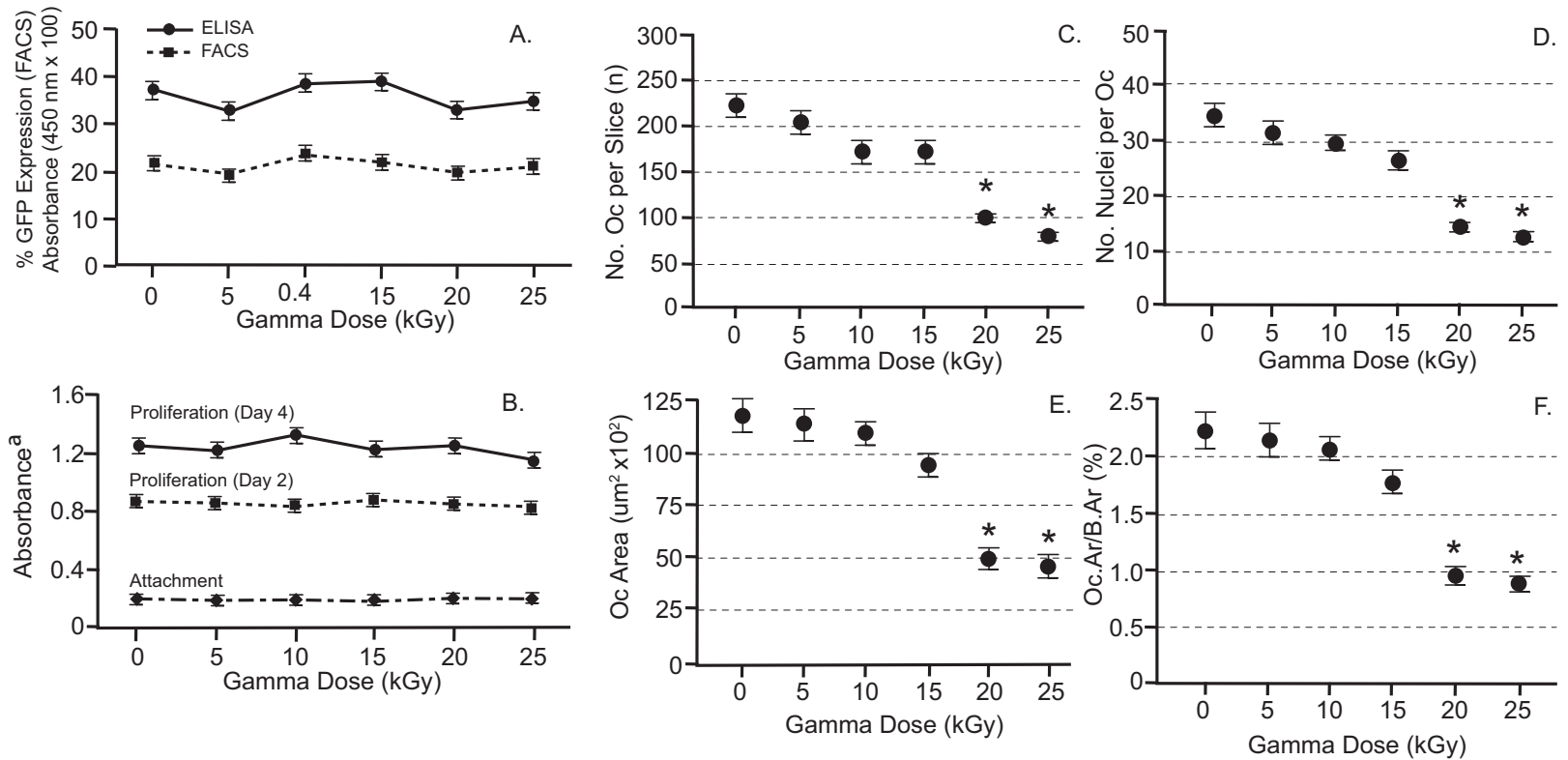

A.
ELISA
FACS
% GFP Expression (FACS)
Absorbance (450 nm x 100)
Gamma Dose (kGy)
B.
Proliferation (Day 4)
Proliferation (Day 2)
Attachment
Absorbance[a]
Gamma Dose (kGy)
C.
No. Oc per Slice (n)
Gamma Dose (kGy)
D.
No. Nuclei per Oc
Gamma Dose (kGy)
E.
Oc Area (um² x10²)
Gamma Dose (kGy)
F.
Oc.Ar/B.Ar (%)
Gamma Dose (kGy)

Figure 2

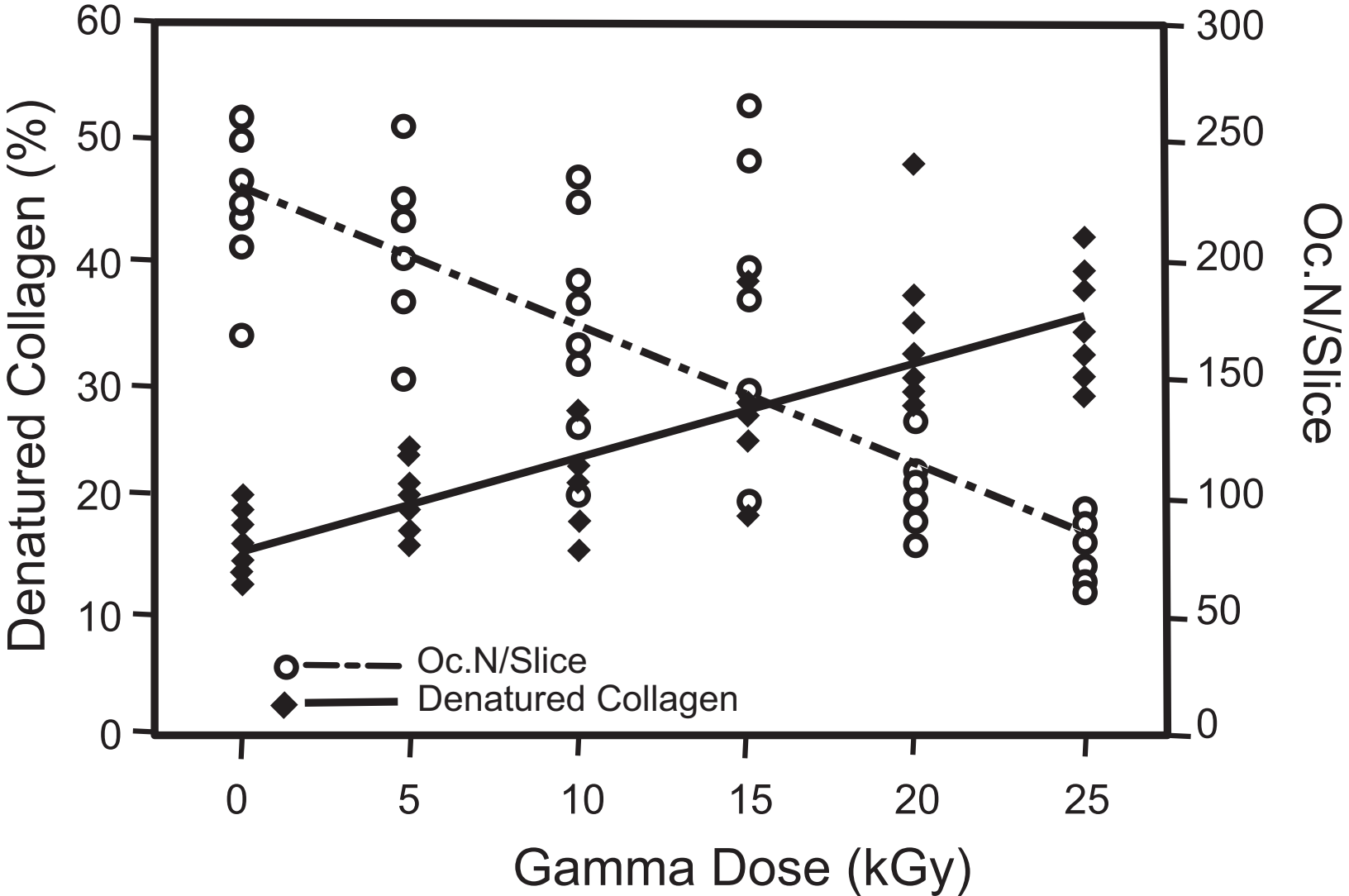
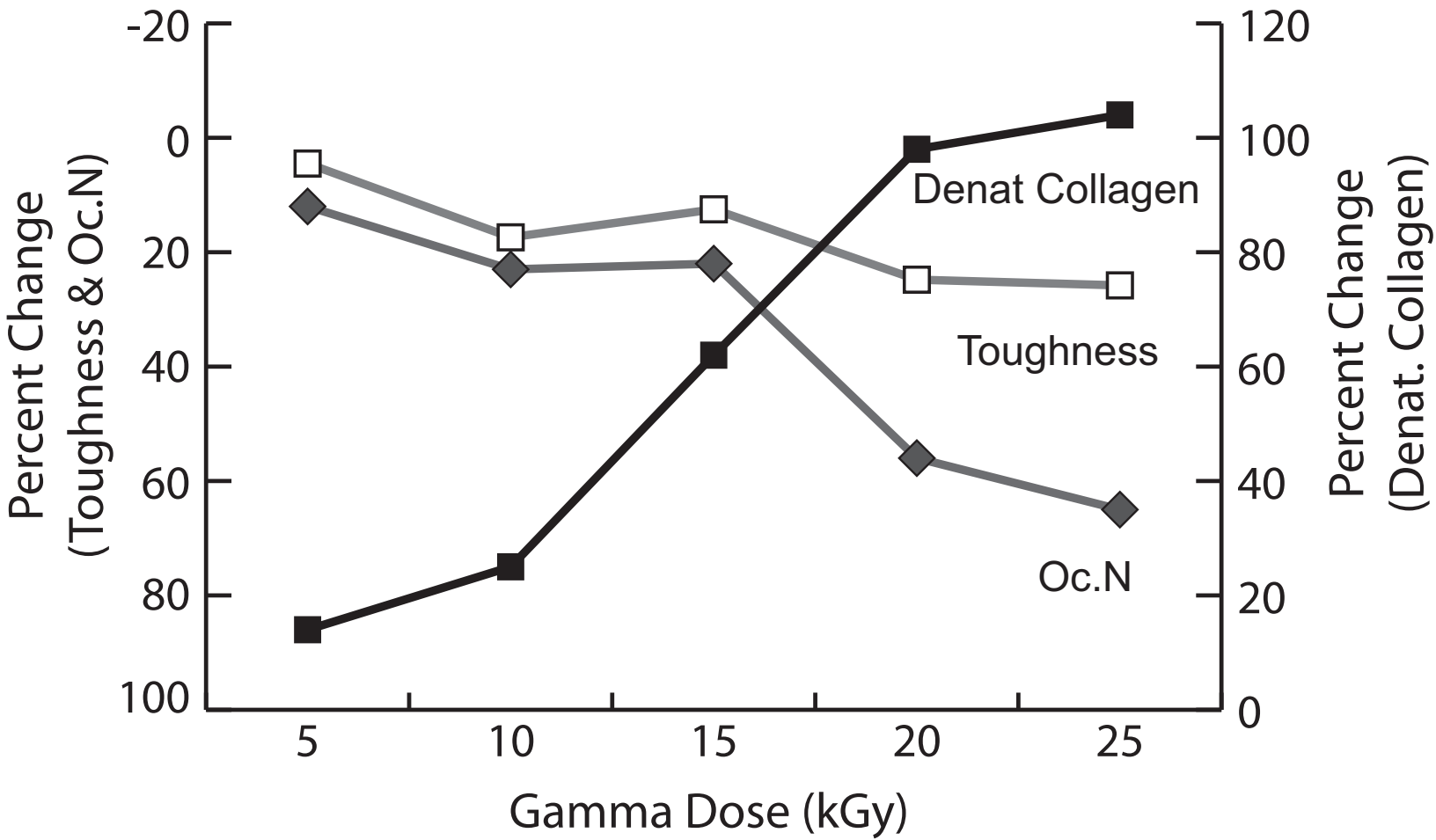

60
50
40
30
20
10
0
Denatured Collagen (%)
300
250
200
150
100
50
0
Oc.N/Slice
Oc.N/Slice
Denatured Collagen
0
5
10
15
20
25
Gamma Dose (kGy)

Figure 3


-20
0
20
40
60
80
100
120
100
80
60
40
20
0
5
10
15
20
25
Percent Change
(Toughness & Oc.N)
Percent Change
(Denat. Collagen)
Gamma Dose (kGy)
Denat Collagen
Toughness
Oc.N