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Bone embrittlement and collagen modifications due to high-dose gamma-irradiation sterilization

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ABSTRACT

Bone allografts are often used in orthopedic reconstruction of skeletal defects resulting from trauma, bone cancer or revision of joint arthroplasty. γ-Irradiation sterilization is a widely-used biological safety measure; however it is known to embrittle bone. Irradiation has been shown to affect the post-yield properties, which are attributed to the collagen component of bone. In order to find a solution to the loss of toughness in irradiated bone allografts, it is important to fully understand the effects of irradiation on bone collagen. The objective of this study was to evaluate changes in the structure and integrity of bone collagen as a result of γ-irradiation, with the hypothesis that irradiation fragments collagen molecules leading to a loss of collagen network connectivity and therefore loss of toughness.

Using cortical bone from bovine tibiae, sample beams irradiated at 33 kGy on dry ice were compared to native bone beams (paired controls). All beams were subjected to three-point bend testing to failure followed by characterization of the decalcified bone collagen, using differential scanning calorimetry (DSC), hydrothermal isometric tension testing (HIT), high performance liquid chromatography (HPLC) and gel electrophoresis (SDS-PAGE). The carbonyl content of demineralized bone collagen was also measured chemically to assess oxidative damage. Barium sulfate staining after single edge notch bending (SEN(B)) fracture testing was also performed on bovine tibia bone beams with a machined and sharpened notch to evaluate the fracture toughness and ability of irradiated bone to form micro-damage during fracture.

Irradiation resulted in a 62% loss of work-to-fracture ($p \leq 0.001$). There was significantly less micro-damage formed during fracture propagation in the irradiated bone. HPLC showed no significant effect on pentosidine, pyridinoline, or hydroxypyridinoline levels suggesting that the loss of toughness is not due to changes in these stable crosslinks. For DSC, there was a 20% decrease in thermal stability ($p < 0.001$) with a 100% increase ($p < 0.001$) in enthalpy of denaturation (melting). HIT testing also showed a decrease in thermal stability (20% lower denaturation temperature, $p < 0.001$) and greatly reduced measures of collagen network connectivity ($p < 0.001$). Interestingly, the increase in enthalpy of denaturation suggests that irradiated collagen requires more energy to denature (melt), perhaps a result of alterations in the hydrogen bonding sites (increased carbonyl content detected in the insoluble collagen) on the irradiated bone collagen.

Altogether, this new data strongly indicates that a large loss of overall collagen connectivity due to collagen fragmentation resulting from γ-irradiation sterilization leads to inferior cortical bone toughness. In addition, notable changes in the thermal denaturation of the bone collagen along with chemical indicators of oxidative modification of the bone collagen indicate that the embrittlement may be a function not only of collagen fragmentation but also of changes in bonding.

Introduction

Bone allografts are used in orthopedic reconstruction of defects due to trauma, bone cancer or revision of joint arthroplasty. In the United States, there are over 1.5 million allograft transplants each year [1] and of these, roughly 450,000 are bone allografts [2]. There are around seventy thousand allograft transplants each year in Canada [3]. A major concern of allograft use is disease transfer and it has become

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common for tissue banks to use allograft sterilization techniques in order to reduce or eliminate pathogen transfer from donor to recipient. Sterilization with γ-irradiation in the 25–35 kGy dose range is widely used because it can effectively eliminate most bacteria, viruses, and fungi. This sterilization is important for the safety of the patient but it degrades the mechanical properties of the bone [4–6] and, in one clinical study, leads to a doubling of the fracture rate of implanted allografts [7]. Irradiation is thought to damage the bone collagen, which is largely responsible for toughness and resistance to fracture [8].

Bone is an intricate material with a complex hierarchical structure. It is a biological composite made up of strong and tough collagen fibers filled with stiff, hard mineral crystals (hydroxyapatite). Type I collagen makes up 90% of the organic matrix of bone [9]. While mineral plays an important role in determining bone stiffness and yield strength, the post-yield properties of bone (such as ultimate strength, toughness and even fracture toughness) are thought to rely highly on an intact collagen network [10,11]. The connectivity of the collagen network, which is influenced by the structure of the triple helices, stabilizing hydrogen bonding, and covalent intermolecular crosslinking, plays an important role in the toughness of bone [12]. Toughness is defined as the ability of a material to absorb energy before fracturing. Fracture toughness is similar but distinct, as it is the ability of a material containing a crack or defect to resist fracture. A study by Zioupos et al. suggests that toughness in bone comes from the natural ability of bone to form stable microdamage (micro-cracking) during deformation [13]. They found that when cortical bone samples were tested in tension at high strain rates there was no time to form micro-cracks before fracture. Furthermore, strong correlations between the amount of microdamage formation, post-yield toughness and strain were observed. Smaller scale pseudo-plasticity mechanisms are thought to include cracking of mineralized fibrils, inter fibrillar sliding, and perhaps molecular uncoiling of tropocollagen [14]. Water is the third major component of bone. Nyman et al. showed that drying bone, even at low temperatures (with between 12 and 24% loss of water by volume), can significantly reduce work-to-fracture (a measure of toughness) which they suggest is due to loss of stabilizing hydrogen bonding in the collagen network and between collagen and then mineral [10].

The mechanical properties of bone suffer greatly from the effects of irradiation. It has been widely demonstrated that the mechanical properties of irradiated bone, particularly the post-yield properties, are significantly inferior to those of non-irradiated bone [4,8,15]. This includes toughness and fracture toughness; properties attributed to the collagen component of bone [5,6,15]. Interestingly, Currey et al. demonstrated early on that a standard dose of around 30 kGy of irradiation decreased deformation-induced micro-crack formation and at around 90 kGy deformation-induced micro-damage formation was almost eliminated [4].

Irradiation is thought to disrupt the collagen network by causing a breakdown in the peptide backbone of the collagen molecules [8,16]. The majority of damage is thought to result from the radiolysis of water molecules, which creates free radicals that attack collagen molecules, thus changing their chemical structure [2,8], breaking peptide bonds and altering amino acids. It has been shown that treating bone with a chemical free radical scavenger during the irradiation process decreases the deleterious effect of irradiation [2], which supports the theory that oxidative damage of collagen molecules is a major mechanism of radiation damage in bone. Akkus et al. demonstrated that irradiation sterilization of intact cortical bone leads to fragmentation of the pepsin-soluble fraction of bone collagen [2]. However, they did not evaluate the nature of the entire collagen network within the bone specimens and therefore the nature of the pepsin-insoluble fraction, which is more heavily cross-linked and load bearing, was not evaluated. In fact, the authors of this present study are not aware of any previous studies that have tested the nature of irradiated bone collagen network as a whole. Fortunately, hydrothermal isometric tension testing is an option for evaluating the stability and connectivity of the collagen network [17,18].

Many investigators have reported embrittlement of bone due to γ-irradiation, yet the underlying mechanisms are not completely understood. Since collagen is a major structural component of bone and lends itself to ultimate strength and toughness, the objective of this study was to evaluate changes to cortical bone mechanical properties as a result of γ-irradiation and to more completely characterize the resulting collagen damage in order to better understand the mechanisms that lead to the γ-irradiation-driven embrittlement of cortical bone. The hypothesis was that γ-irradiation sterilization leads to dramatic loss of bone collagen network connectivity (a function of crosslinking and chain length evaluated with hydrothermal isometric tension testing) paralleling the loss of toughness and of the bone's ability to form micro-cracks during deformation. Furthermore, our characterization techniques revealed new findings regarding irradiation-modified bone collagen at the molecular level.

Materials and methods

Sample preparation

Eight tibiae from steers (aged approximately 1.5 to 2 years old) were obtained immediately after slaughter from a local abattoir and kept frozen (−20 °C) for 3–10 days until dissection. Frozen bones were thawed and stripped of all soft tissue (muscle and fat). The periosteum was scraped from the bone surface using a surgical scalpel. Using a band saw, bones were cut into blocks approximately 70 mm × 25 mm × 6 mm. These blocks were kept frozen while wrapped in saline soaked gauze. The location (distal anterior or distal posterior) and animal were noted. We have observed that the bone in both locations is plexiform with the occasional occurrence of secondary osteons. Later, each block was cut into rectangular beams with the length aligned with the

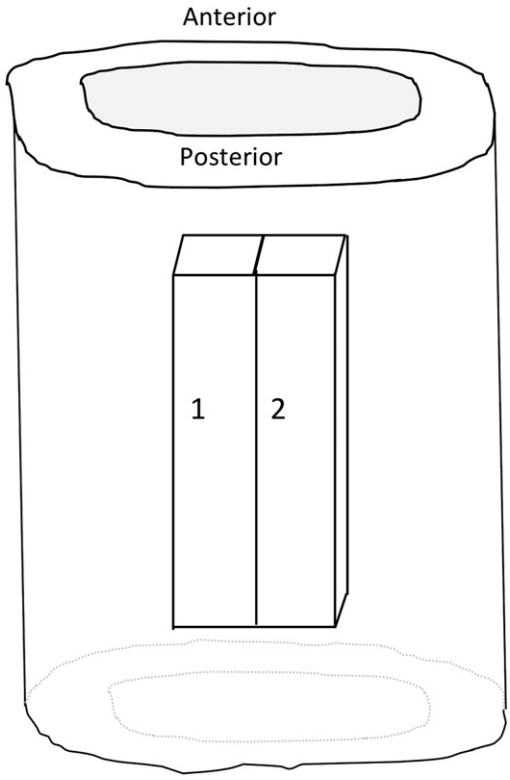

Fig. 1. Bone beam preparation schema. Matched pairs were cut from adjacent bone.