STABILIZATION OF IRRADIATED ALLOGRAFTS VIA CROSSLINKING AND FREE RADICAL SCAVENGING

by

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ABSTRACT OF THESIS

Stabilization of Irradiated Allografts via Crosslinking and Free Radical Scavenging

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Currently, the risk of disease transmission from allografts has been reduced through a system of screening methods organized by tissue banks. Terminal sterilization is possible with the use of ionizing irradiation, which has shown high efficiency for neutralizing pathogens. Unfortunately, irradiation is known to cause chain scission and crosslink degradation in collagen, leading to weakened mechanical properties and increased susceptibility to enzymatic digestion.

Two methods of stabilizing allografts against the effects of irradiation were studied in this research. These methods included crosslinking via EDC or glucose, and free radical scavenging using mannitol, ascorbate, or riboflavin. The overall objective of this study was to assess the protective ability of each treatment in the presence of increasing irradiation dose based on mechanical properties and enzyme digestion. These studies also investigated the influence of tendon water content, and combination of crosslink and scavenging methods after exposure to irradiation. Our hypothesis was that crosslinking and free radical scavenging would aid in maintaining mechanical integrity and enzymatic resistance after gamma or ebeam irradiation.

In general, crosslinking and free radical scavenging improved the mechanical properties and collagenase resistance of irradiated tendons. Glucose crosslinked tendons irradiated at 25 kGy were comparable in terms of strength to native tendon. Similarly, at 50 kGy EDC crosslinked tendons were comparable to untreated tendons irradiated at 25 kGy. Ascorbate and riboflavin were successful at protecting mechanical properties especially at 25 kGy. Most noteworthy were the combined treatments irradiated at 50 kGy, which matched native tendons mechanically and were highly resistive to collagenase.

These treatments were unable to completely maintain properties at 50 kGy, which is closer to a clinically useful dose. The majority of treatments displayed improvements at 25 kGy. If allografts could be successfully stabilized from damage, ionizing irradiation could ensure not only disease free tissues, but also faster availability.

PREFACE

The Orthopaedic Research Laboratory at Robert Wood Johnson Medical School has developed years of expertise in the field of musculoskeletal tissue engineering. My colleagues are currently working on other projects involving anterior cruciate ligament and menisci replacement. My research, which addresses the issues behind allograft safety and sterilization, only represents a sample of the work done in our lab.

ACKNOWLEDGEMENTS

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I have also made a lot of friends over the years at Rutgers who helped make New Jersey a home away from home. Finally it would be unthinkable to forget my own family. Thanks to everyone that supported and encouraged me throughout graduate school.

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1 Introduction

1.1 Allografts, disease transmission, and ionizing irradiation

Surgical intervention has always been the common treatment method for severe musculoskeletal injuries. Often these procedures require replacement of damaged tissue with a graft. Current options are autografts, tissue harvested from the patient, or allografts, same species tissue harvested most commonly from cadavers. In the past decade, there has been an increase in preference for using allografts, which is reflected by the increase in allograft implants from 650,000 in 1999[1], to 1.5 million in 2007[2]. This figure implies there are obvious advantages to the use of allografts, however there are also several disadvantages associated. The incentives to use allografts include reduced tissue morbidity and greater abundance, allowing for better anatomical matching[3,4]. Among dangers involved with implantation of allografts is an antigen mediated immune response. However, the main danger associated with using cadaver tissue is the potential for disease transmission[5-7]. This can lead to prolonged inflammation, impaired remodeling, and ultimately graft failure[8,9] or even fatality[6].

Tissue banks have developed a system of preventative measures to improve safety. Deterrence of disease transmission has been approached by a combination of several procedures. The simplest of these is an extensive investigation into donor medical history[6,7,10]. Additionally, grafts are commonly subjected to screening assays aimed at identifying pathogens[6,7,10]. Finally, they

are subjected to aseptic processing and handling upon harvest[4,7]. However these protocols are not without their limits and cannot completely ensure safety against disease transmission[10]. Donor medical histories have the possibility of being incomplete or incorrect, screening assays are highly selective and will not encompass all diseases, and aseptic processing will only defend against post harvest contamination. These preventative measures are even more vulnerable to late infections by bacteria or viruses that occur just prior to death. This is a period of opportunity, referred to by many as the “silent window”, for pathogens to pass undetected and infect the recipient[10]. The pathogen will pass though patient history investigation because symptoms may not arise prior to death. Screening assays may not catch it, as it is a recent infection and likely below the threshold for detection[10]. This problem can be avoided using high dose sterilization via ionizing irradiation, which has shown to be extremely capable at neutralizing bacteria and viruses[11]. Furthermore ionizing irradiation is a simple and effective process that can easily be regulated among tissue banks, which will result in accelerated distribution. However irradiation of collagen-based materials has been known to result in loss of mechanical stability[10,12,13], and increased rate of degradation by of proteolytic enzymes[14]. These irradiation effects are often codependent, and are influenced by damage to collagen molecules. The function of an allograft is to serve as a temporary but functional scaffold, bridging necessary time to allow neotissue formation[15]. The research presented in this dissertation takes aim at counteracting the negative effects of ionizing irradiation

on collagen. Successful stabilization of allografts allows terminal sterilization via ionizing irradiation to be used to ensure safe distribution.

1.2 Types of Sterilization

Tissue banks often include sterilization procedures, but these have not become standard due to their adverse effects[10]. There has not been a drastic change in sterilization methods from the past to the present. The two main types of sterilization that have been utilized throughout the history of allograft harvesting are ethylene oxide and ionizing irradiation[4].

1.2.1 Ethylene Oxide

Ethylene oxide (EO) has been used as a sterilizer for medical devices that are sensitive to heat or moisture[4]. These are cases where conventional autoclaving is not an option. The need for 'cold' sterilization was motivated by more frequent use of single-use instruments, often made of plastic or rubber[4]. A 1980 review of EO use initially deemed it an ideal sterilant for bone and soft tissue allografts[16]. Studies have compared EO sterilized allografts to frozen controls and have found no differences in mechanical properties[16,17]. This has been motivation for use of EO sterilization for allografts[4,17]. However other studies conducted on EO sterilized allografts introduced concerns regarding its effectiveness to ensure sterility as well as its effects on graft performance post-implantation[4]. Ethylene oxide is used in a gaseous state at room temperature, and sterilization occurs at points of contact[4]. Adequate and uniform penetration

was questioned when dealing with dense tissues like cortical bone[16]. Proper exposure and aeration times have been suggested for management of these problems[4], however increased processing time has its own obvious disadvantages. More importantly, it became evident that residual EO caused cytotoxicity and inflammation[18]. A study conducted by Jackson et al. cataloged patients receiving anterior cruciate ligament (ACL) reconstructions with bone-patellar-bone allografts, and 7 of 109 patients required removal of grafts as a result of persistent inflammation. This study attributed graft failure to residual EO and byproducts, and found that the effects subsided following removal[18]. According to the American Associated of Tissue Banks, EO was used in two thirds of national tissue banks in 1987-1988. In a follow up survey conducted in 1992, the use of EO had dropped off and the two thirds of tissue banks had begun using irradiation as an alternative[4].

1.2.2 Ionizing Irradiation

The two main forms of sterilization classified as ionizing irradiation currently used are gamma and electron beam (ebeam) irradiation[11]. Gamma irradiation has been a mainstay in tissue bank procedure[19], and possesses a vast pool of previous research. Electron beam technology has also been well studied and well established, however it has not been used extensively for the purpose of tissue sterilization. As a result, the studies discussing the effects of ebeam irradiation on allografts have been sparse. Both of these methods are much faster than EO, are extremely effective steriliants, and possess no associated

cytotoxicity[8,20]. However the effects on collagen are a main concern and have not been overcome.

Both types of irradiation result in the exposure to high energy, and the absorbed dose is measured in units of greys. One grey (Gy) is equal to one joule of energy applied to one kilogram of mass. Dose needed for allograft sterilization is typically on the order of kGy[4].

Gamma Irradiation

Cobalt 60 and Cesium 137 are the major sources for gamma irradiation[11]. Recently however, the use of cobalt 60 has become almost exclusive for industrial purposes[11]. Cobalt 60 is derived from neutron absorption in its natural stable state cobalt 59. Upon transformation to radioactive cobalt 60, the atom begins to decay to become a stable nickel 60 atom[11] (Fig. 1-1).

In the case of gamma irradiation, gamma rays are released when the nucleus of an atom returns from an excited state to ground state. Gamma rays are photons with energies of 10keV to 10 MeV, and photons are particles of electromagnetic radiation[11]. Cobalt 60 sources emit two photons (gamma rays) at energy levels of 1.17 and 1.33 MeV during beta decay transition to Nickel 60[11] (Fig. 1-1). Gamma rays are ideal for sterilization of allografts because of high penetrability and processing speed[11]. Gamma irradiation is a one step process, and unlike EO does not require aeration steps to remove residuals.

1) $Co_{27}^{59} + n_0^1 \xrightarrow{absorption} Co_{27}^{60}$

2) $Co_{27}^{60} \xrightarrow{betadecay} Ni_{28}^{60} + e_{-1}^0 + 2\gamma$

Figure 1-1 Source of gamma irradiation energy
Beta decay of Cobalt 60 releases two gamma rays.

Electron beam irradiation

Electron beam (ebeam) irradiation was first used for medical devices in the 1950s for sterilization of sutures[21]. Ebeam irradiation uses an accelerated stream of electrons as its radiation source[22]. The electrons are released at high energies between 5 and 10 MeV, and are accelerated through a charged field created by a positively charged cathode and negatively charged anode[22] (Fig. 1-2). Ebeam irradiation does not have the penetrability of gamma irradiation, but is benefited by a higher energy source and a higher dose rate. At a higher dose rate, ebeam irradiation can complete sterilization in seconds whereas completion of gamma irradiation would be on the order of hours[22]. Ebeam irradiation has roughly a .5 cm penetration through a density of water per MeV[22]. Comparatively, thickness of 10 cm of water will reduce the intensity of gamma rays by 50%[22]. Ebeam irradiation boasts speed but lacks penetration of gamma irradiation. Furthermore, ebeam irradiation is conducted along a conveyor belt and can quickly alter intensity as dose requires[21]. For sterilization of thin soft tissue, penetration is adequate for both types of irradiation.

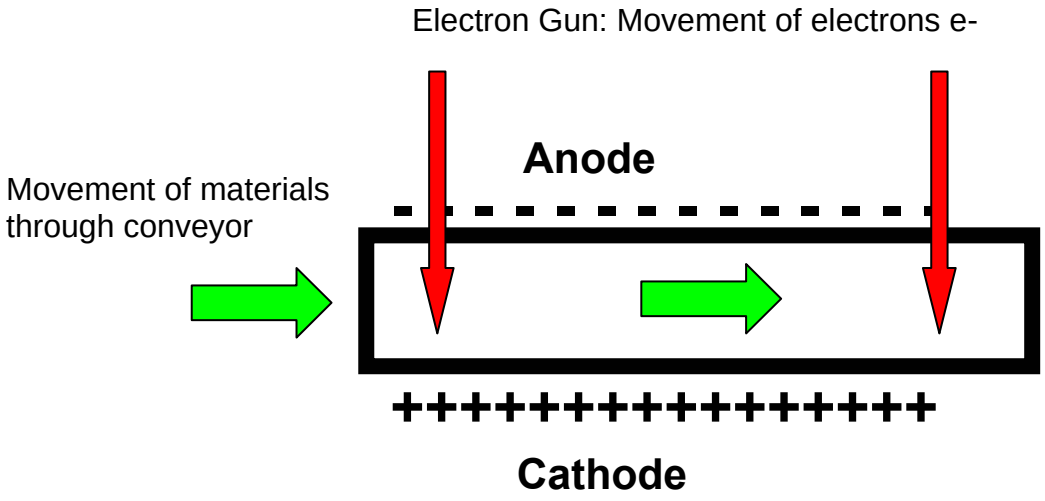


Figure 1-2 Schematic of ebeam irradiation

Materials to be sterilized move through a conveyor belt and under an electron beam gun that releases electrons that are accelerated through a gradient of charge. Materials are irradiated and sterilized by electrons at energies between 5-10 MeV[22]. The convience with this set up is the ability to alter the dose for materials on the conveyor belt.

1.2.3 Bacteriacidal and viricidal effects of irradiation

Damage to bacteria and viruses resulting from sterilization occur via two pathways: direct and indirect. Direct damage involves scission of bonds as a result of impact with the high energy particles. However, the indirect result of irradiation is the primary source of damage[11]. This occurs through the irradiation of surrounding oxygen and water resulting in the formation of free radicals[11] and their effects on chemical structures. These free radicals are highly volatile and subsequent reactions can perpetuate the formation of more free radicals. Chemical modifications, the breakdown and formation of bonds, will often lead to loss of biological function. The critical example being bacterial or viral DNA[11],

which results in the loss of the ability to generate vital proteins. The damage will occur throughout the cell, undoubtedly causing premature cell death[23]. These results are desired for efficient sterilization, but unfortunately collagen, the major component of musculoskeletal tissues, is susceptible to irradiation damage[10,13,14,24]. This is the main concern faced when sterilizing collagen allografts[12].

1.2.4 Environmental effects on irradiation

Many environmental factors can influence sterilization, and they must be well regulated in order to accurately make comparisons between results. It is believed that the discrepancies or conflictions among results in the literature are the cause of varying environmental conditions[10]. The most important factor determining the effectiveness of irradiation is the presence of oxygen or water. Irradiation of oxygen generates many oxygen derived radicals collectively known as ROS (reactive oxygen species) (Fig. 1-3)[11]. These molecules by name are highly reactive and perpetuate the formation of more radicals. ROS are most commonly formed by irradiation of atmospheric oxygen and water present within tissue or cells. For the duration of this paper these will be referred to generally as free radicals. Regarding discussion of free radical damage, other radicals such as organic radicals also cause damage [25].

To a slighter extent, irradiation is also effected by temperature. Free radical migration is impeded in frozen materials[11,26]. When comparing materials irradiated in a frozen versus aqueous environment, irradiation effects are more

pronounced for materials in solution. All these factors (Table 1-1) must be considered when determining irradiation dose for sterilization.

$$H_2O \xrightarrow{Irradiation} H_2O^+, e_{aq}^-, OH^-, O_2^-, HO_2^-, H_2O_2, H_3O^+$$

Figure 1-3 Irradiation of water

Reactive oxygen species that can be potentially generated from the irradiation of water[11]. This figure is not a chemical reaction.

Table 1-1 Environmental/Sterilization factors influencing pathogen resistivity.

Table adapted from Block[11]

Factors Increasing Resistivity	Factors Decreasing Resistivity
Frozen materials	**Water or oxygen presence**
Protectors (free radical scavengers ie Thiourea)	**Sensitizers (free radical generators ie hydrogen peroxide)**
High dose rate	**Low dose rate**

1.3 Irradiation of Collagen

Collagen is highly susceptible to free radicals[27-29], which are generated by the irradiation of the high abundance of water molecules associated with soft tissue allografts. Among these include highly reactive hydrogen peroxide, superoxide anion, and hydroxyl radicals[11]. Direct impact by photons causes scission of bonds in collagen backbone chains[12,14,24]. Indirect effects of free

radicals are believed to cause denaturation, but also target crosslinks[12-14,24]. Salehpour et al. has shown that hydroxypryidinium crosslink density (the intermolecular crosslinks in collagen) is decreased with irradiation of goat patellar tendon[13]. In addition to crosslinks, free radicals such as superoxide anion $-O_2$, have shown preference to cleave collagen helices[28]. Exact mechanisms causing collagen damage have not been elucidated.

1.3.1 Structure of collagen

The collagen molecule is the basic unit comprising all musculoskeletal grafts, and therefore irradiation effects on collagen allografts can be best studied by analyzing collagen. Collagen itself is constructed of three intertwined alpha helical units together known as tropocollagen[30] (Fig. 1-4). Each chain is about 1000 amino acids in length, and the tropocollagen molecule is roughly about 300 nm long[30]. Each alpha helix is surrounded by a thin layer of proteoglycans and glycosaminoglycans[30]. The amino acid composition of collagen consists of sequences of amino acids in collagen occur in repeating units of Gly-X-Y, where X and Y represent variable amino acids[30]. However both proline and hydroxyproline compose 30% of collagen[30]. Glycine is the smallest of amino acids, and molecules with large glycine content are generally amorphous and flexible. Conversely, proline and hydroxyproline contain double bonded rings and are both large and rigid[30]. Tropocollagen units are bundled and interlocked in a quarter stagger assembly in collagen fibrils, which are the base of a rising hierarchy of structures comprising native tendons[31] (Fig. 1-5). These quarter

stagger connections or crosslinks result in slipping of tropocollagen units, and dislocation of fibrils[31].

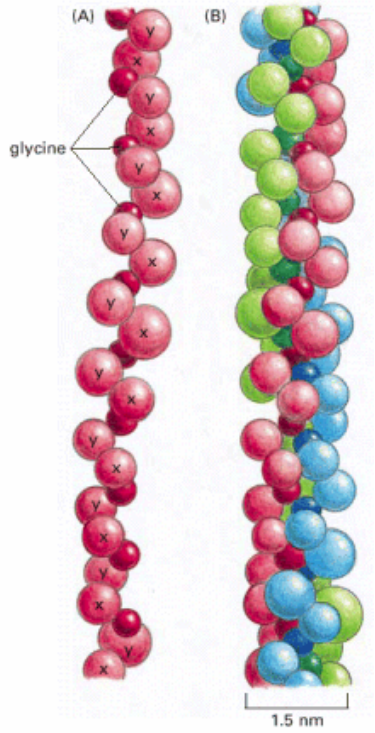


Figure 1-4 Tropocollagen molecule

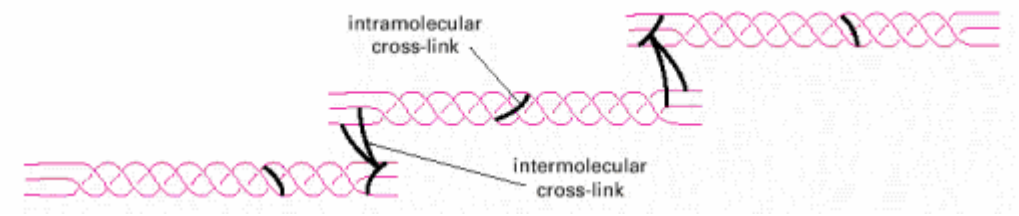
A) One alpha chain strand displaying the –X-Gly-Y- sequence of collagen molecules. B) Three chains together make the tropocollagen structure of collagen [32].

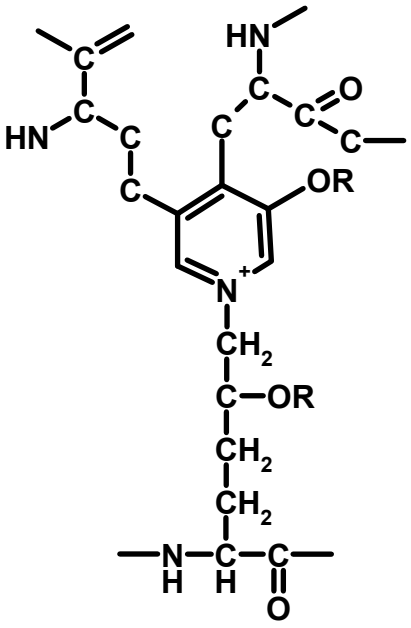
1.3.2 Collagen crosslinks

Collagen is naturally crosslinked within (intramolecular) and between (intermolecular) tropocollagen subunits. Intramolecular crosslinks are formed by an enzyme catalyzed conversion and aldol condensation between two lysine residues[30] (Fig. 1-6). Intermolecular crosslinks are formed from a lysine and two hydroxylysine residues, known as the hydroxypyridinium structure[33] (Fig. 1-5). Native crosslinks provide musculoskeletal tissues like tendon and ligaments high tensile strength. Surface crosslinks are also believed to reduce antigenicity in

implanted allografts[34,35]. Heightened immune responses are triggered by foreign surface markers, and can result in graft rejection. Adding crosslinks to the allograft surface and throughout can aid in masking or enveloping these antigens, and preventing them from signaling a response[36].

When studying the effects of irradiation on allografts, it was mentioned that the result is a function of effects on collagen. It has been observed that ionizing irradiation of allografts results in the following coupled effects: loss of mechanical properties[10,12,13] and reduced resistance to enzymatic degradation[14], which can ultimately lead to graft failure. These effects are known to be related to collagen content and crosslink density, which are susceptible to free radical interactions.





Intermolecular Crosslink Hydroxypyridinium Structure

Figure 1-5 Intermolecular crosslinks

Staggered hydroxypyridinium crosslinks formed between lysine and two hydroxylysine residues. These intermolecular crosslinks provide collagenous materials with great tensile strength[32].

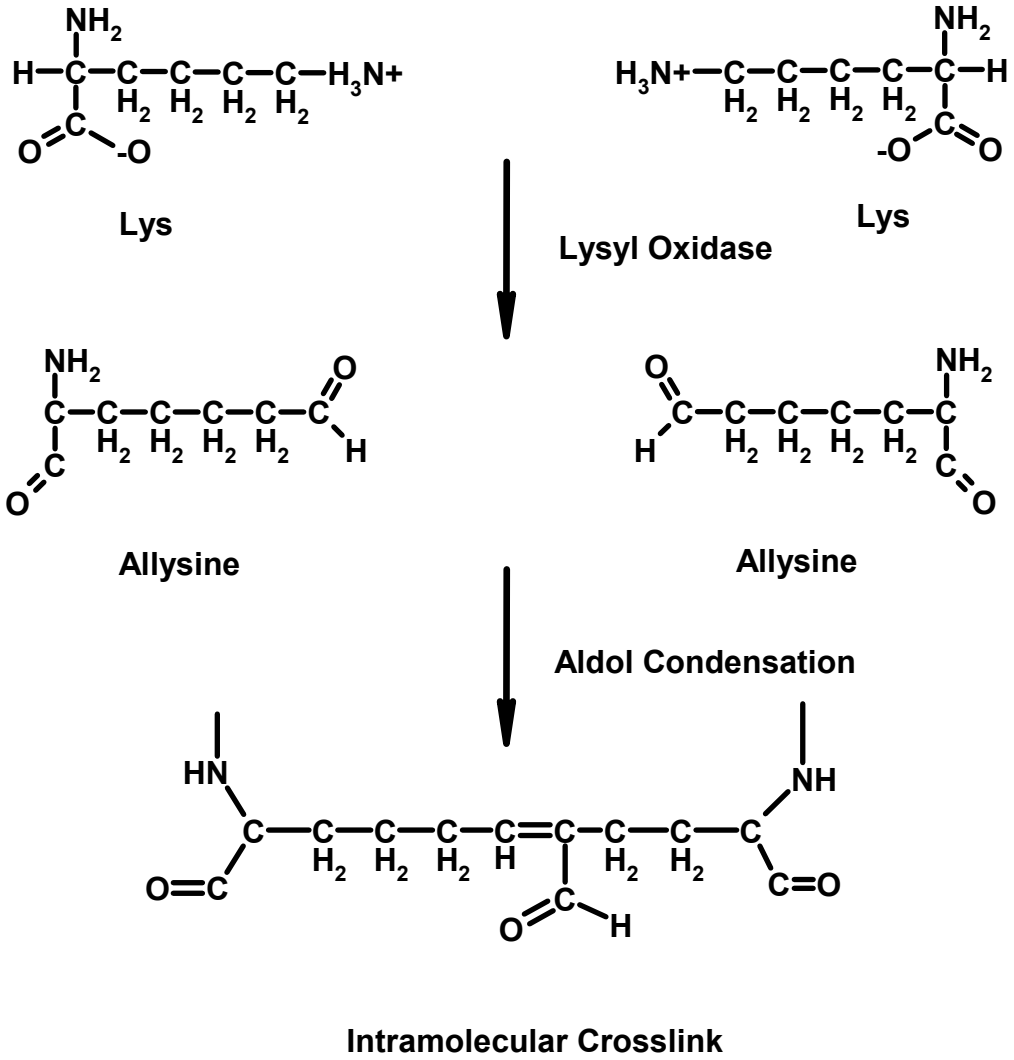


Figure 1-6 Intramolecular crosslinks

Intramolecular crosslinks are formed aldol condensation of enzymatically transformed lysine residues.

1.3.3 Loss of Mechanical stability

The reduction in mechanical properties after gamma irradiation of tendon allografts has been well established[10,11,13]. Correlations have been made between mechanical properties and crosslink density[37]. Studies have observed both lower mechanical properties and hydroxypryidinium crosslinks in tendons after irradiation[13]. Furthermore, increases in strength and modulus have been observed in collagen allografts crosslinked by gluteraldehyde[38]. The ability of irradiated allografts to bare functional loads is among the primary concerns when using this method of sterilization. Initial strength is a necessary characteristic for allografts. They must be handled during surgery and maintain stability during the course of the healing process[39]. Several studies have reported loss of mechanical integrity at doses over 25 kGy[19,40]. Although this finding has been flexible most likely due to variability in processing conditions. It has been agreed upon that this reduction is dose-dependent[13]. Specifically increasing doses of irradiation resulted in lowered properties[13].

1.3.4 Enzymatic degradation and remodeling

While initial mechanical strength is an important allograft property, it does not predict post implantation survival. Furthermore, premature degradation in vivo is coupled with the loss of functional strength[41]. Chain scission and free radical alteration of crosslinks increase the susceptibility of collagen to enzymatic breakdown[20]. This occurs because damage to crosslinks and three-dimensional structure allows enzymes easier access to cleavage sites[42]. This ultimately leads

to quicker dissolution time. Post implantation integrity is important to allow time for cell attachment and neotissue formation[20]. Ideally, these cells will remodel the allograft and initiate incorporation. Degradation of crosslinks and breaking of peptide bonds by irradiation has shown to result in shrinkage of collagen sponges, which impeded cell infiltration in an in vitro study[20].

1.4 State of allograft sterilization

Currently, standard sterilization protocols are not regulated among tissue banks. The International Atomic Energy Agency (IAEA) has made the recommendation to use 25 kGy as a standard dose, which is followed by many tissue banks worldwide[5]. Sterilization doses frequently range from 10 kGy to 35 kGy[4], and some tissue banks chose to rely solely on screening methods. Although to use ionizing irradiation for terminal sterilization, doses would likely have to be much higher. A consensus among several studies has been made declaring 25 kGy not high enough to neutralize more resistant bacteria and viruses such as human immunodeficiency virus (HIV)[13,40,43]. Meanwhile, at higher doses weakening and degradation of irradiated allografts are even more enhanced[13]. Methods of fully counteracting strength reduction and protease degradation have not yet been developed. Therefore high dose irradiation of allografts as not been a feasible option for terminal sterilization.

1.5 Hypothesis and approach

In order to reverse the effects of irradiation, a logical approach is to address the primary causes: chain scission and the generation of free radicals by irradiation of water and oxygen in soft tissues. The combined result is damage to collagen structure and crosslinks[13]. This consequently leads to weakened allografts[13] and enhanced degradation to proteases[14]. **We hypothesize that free radical scavenging and crosslinking, which are aimed at protecting collagen nativity and crosslink density, will translate to improved mechanical properties and degradation after irradiation.** The specific purpose of this research is to conduct preliminary studies among a large pool of treatment groups and identify those that provide successful protection. This is due to the lack of scientific knowledge and novelty regarding the use of these techniques for stabilization in the presence of irradiation.

1.6 Free radical scavengers

Agents that serve to protect against damage caused by irradiation are considered radioprotectants, which include crosslinkers and free radical scavengers or antioxidants. Free radicals are characterized by unpaired electrons[23], and scavengers stabilize free radicals by gaining or donating electrons thereby preventing undesired reactions. Free radical scavengers are successful because they have stable oxidation or reduction states. Natural scavengers are ideal to use against free radicals, and are advantageous because of their small size and penetrability[12], non-toxicity, and only need to be

present in solution at the time of irradiation[44]. Deactivation of free radicals prevents them from participating in structure altering reactions with collagen. Scavengers have been studied for use against damage from gamma irradiation[44-48]. Although, these studies have been limited to aqueous environments and much lower doses. Assessment of free radical scavenger capability in muscuoloskeletal tissues has only recently begun[10,12]. Furthermore, the exact mechanisms of protection regarding these scavengers is unknown[49]. The free radicals chosen for this study include mannitol, ascorbate, and riboflavin.

1.7 Overview of crosslinking

Crosslinking of collagen has been performed in the past for reducing antigenicity[35], strengthening[33,38,50], and improving degradation resistance[51]. A very common crosslinker used in the past was gluteraldehyde[52,53]. Although highly successful at performing these tasks, it has been found that hydrolysis of crosslinked collagen releases monomeric gluteraldehyde, found to be cytotoxic in vivo[52,53]. More recently, EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) has been studied for use as a collagen crosslinker. EDC has shown to effectively crosslink collagen at optimal concentrations[54] and more importantly possess no cytotoxic effects[52]. Glucose has also been shown to participate in collagen crosslinking and has been studied in association with aging and diabetes[55,56]. Furthermore, in vitro glucose crosslinking has been shown to increase mechanical properties in rabbit tendon[57]. EDC and glucose were chosen for their non-toxicity and effective crosslinking.

1.8 Study Outline

Assessment of success for crosslinking and free radical scavenging treatments was done through analysis of mechanical properties and susceptibility to enzymatic degradation. Irradiation effects were initially studied on untreated native tendon to use as a basis for comparison with treated groups. Mechanical properties were evaluated through tensile testing, and degradability through collagenase and trypsin resistance testing. Additionally, all groups were exposed to both gamma and ebeam irradiation to allow comparison of irradiation types. Effects of irradiation on freeze drying storage were also compared to frozen among untreated groups. This was done to study the influence of water content on effects of irradiation on tendons. Finally, work was begun on combining EDC crosslinking and free radical scavenging in order to potentially obtain benefits of both treatments.

2 Materials

2.1 EDC crosslinking

EDC is a zero order crosslinker that has been heavily investigated as a crosslinker for collagen biomaterials (Fig. 2-1). Previous work from this lab has shown EDC to successfully strengthen collagen constructs and resist degradation from proteolytic enzymes[51,58]. The only byproduct of crosslinking reaction is urea[51], which is water soluble and can be washed away. Also as a zero order crosslinker, crosslinked ends are joined directly with a covalent bond without any residual spacer molecules[51,54].

The mechanism of EDC crosslinking involves formation of an O-acylisourea derivative, which joins carbodiimide with the carboxylic acid groups of glutamic or aspartic acid residues[51,54]. This is followed by the nucleophilic substitution of an amino group, which usually occurs with the E-NH2 group of lysine[51]. Upon substitution, the crosslinking bond is formed and the urea derivative is released[51,54]. The limiting factor of this reaction is the stability of the urea derivative, which is susceptible to hydrolysis and rearrangement[51,54]. The solution to this problem is to introduce N-hydroxysuccinimide (NHS) to the crosslinking reaction. NHS serves to increase crosslinking efficiency by suppressing two side reactions: the hydrolysis of O-acylisourea by water to N-subtituted urea and the original glutamic or aspartic acid carboxyl group, and the other is the rearrangement of the O-acylisourea group to a more stable N-acylurea[51,54]. When NHS is present, O-acylisourea is converted to an NHS-activated carboxylic acid, which is more stable and able to carry on with the

crosslinking reaction[51]. To our knowledge EDC crosslinking of collagen has not been studied for stabilization of allografts against ionizing irradiation.

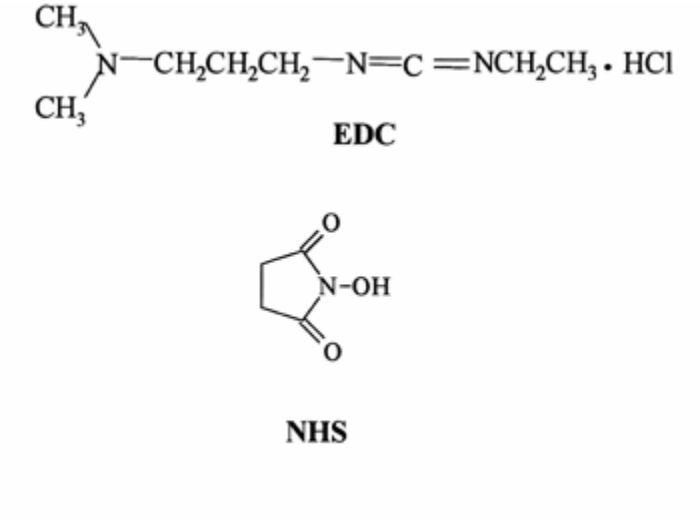


Figure 2-1 Chemical structure of EDC and NHS

EDC crosslinking of collagen results in direct attachment of glutamic and aspartic acid residues without any residual spacers. NHS serves to suppress side reaction to push the crosslinking reaction forward[51].

2.2 Glucose crosslinking

Glucose crosslinking occurs between lysine or hydroxylysine and arginine residues, and is capable of forming both intramolecular and intermolecular crosslinks[59]. Crosslinks are produced through the Malliard reaction, which initiates in the joining of peptide lysine and glucose to form a glucosyl-lysine Schiff base[55]. This step is followed by an Amadori rearrangement, which subsequently undergoes several dehydrations and further rearrangement. The final products are pentosidine, 2-furoyl-4(5)-(2-furanyl)-1H-imidazole (FFI), and carboxymethyllysine (CML), which are three reactive carbonyl compounds that

will contribute to crosslinks in collagen. The progression of this reaction is dependent on the switch of glucose between cyclic and linear conformations[59]. Glucose must be in the linear form to participate in the Maillard reaction[59] (Fig. 2-2).

Previous work has shown that free radicals expedite this crosslinking reaction in collagen films[60,61]. Both ultraviolet (UV) and gamma irradiation derived free radicals were shown to drive glucose crosslinking[60,61]. It is theorized that free radicals encourage the linear form of glucose and thus driving the reaction forward [59]. Therefore unlike EDC pre-crosslinking, glucose crosslinking occurs at the time of irradiation. Free radical generation by ionizing irradiation is known, and it is possible that motivation of crosslinking in tissues is similar to films. Glucose crosslinking using conventional soaks has been performed in rabbit Achilles tendon in the past[57]. After 4 weeks of soaking, significant increases in mechanical parameters were recorded[57]. However the speed of crosslinking using this method is only limited by speed of irradiation. The current study is the first to implement this free radical expedited technique in collagenous tissues.

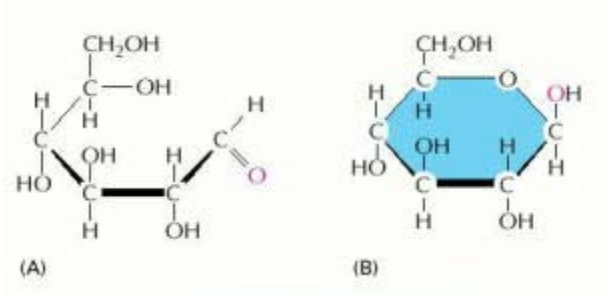
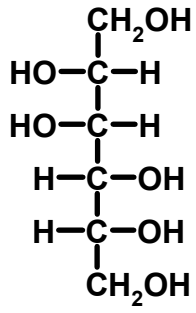


Figure 2-2 Chemical structure of glucose

A) Linear form of glucose B) Cyclic form of glucose[32]. Glucose must be in the linear form to participate in the Malliard reaction[59]. Glucose constantly rotates between both conformations, but superior stability keeps it in the cyclic form longer.

2.3 Mannitol

Mannitol is a water soluble sugar alcohol and is formed by reduction of mannose, an epimer of glucose[62] (Fig. 2-3). There have been several past studies showing the protective effects of mannitol against irradiation derived free radicals[10,63]. Strand breaks in gamma irradiated bacterial DNA was reduced when mannitol was included[64]. However due to the structural complexities of tissues compared to aqueous environments, studies involving irradiated tissues are more comparable. Grieb et al. has shown that a radioprotective cocktail including mannitol and strict processing conditions preserved mechanical properties of human semitendonosus allografts irradiated with gamma irradiation at 50 kGy[10].



D-Mannitol

Figure 2-3 Chemical structure of mannitol

The linear conformation of the sugar alcohol mannitol, which possess free radical scavenging ability.

2.4 Ascorbate

Ascorbate is a water soluble antioxidant and traditionally a good electron donor (reducing agent)[23]. It is the dehydrogenated form of ascorbic acid or vitamin C (Fig. 2-4). Ascorbate can donate one electron yielding monohydroascorbate and once further to dehydroascorbate[23]. Ascorbate reacts rapidly with O_2-, HO_2 radical, and even more rapidly with OH- ions. These oxidation reactions have been studied and confirmed in reactions between ascorbate and free radicals[23], but identification of specific reactions was not done for this study. Similar reduction reactions were believed to allow ascorbate to detoxify organic carcinogens generated from ionizing irradiation in animal studies[23].

The first oxidation state of ascorbate (monohydroascorbate) is stable because of its chemical structure. After an electron is removed, the remaining electron is able to delocalize among oxygen atoms[23]. Molecules with delocalized electrons are stable because movement of electrons allows them to constantly

be as far apart from each other as possible, and thus lowering the potential energy of the molecule and increasing stability.

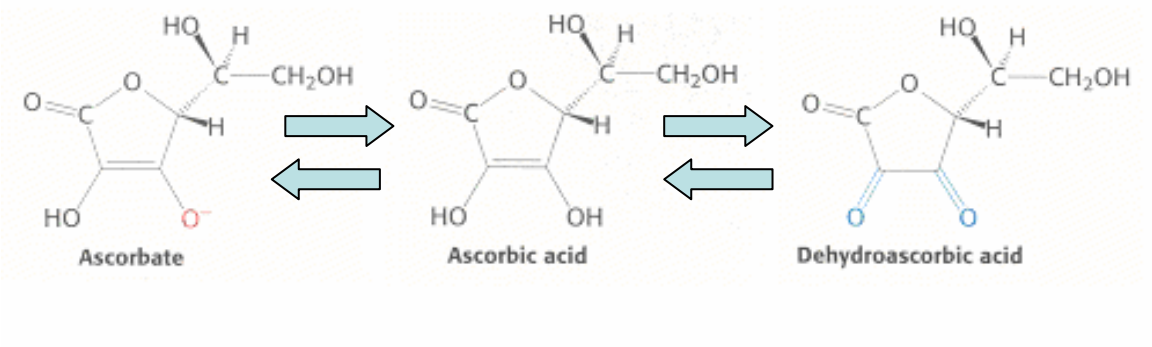


Figure 2-4 Redox reactions involving ascorbic acid

Ascorbate can accept a hydrogen to become ascorbic acid, which then can release two electrons to become dehydroascorbic acid. This reaction can also proceed in the reverse, however monohydroascorbic acid (the intermediate between ascorbic acid and dehydroascorbic acid) is stabilized by resonance [23,65].

The effectiveness of ascorbate to protect against gamma irradiation has been studied in the past. Cai et al. acknowledged the potential for vitamin C to act as a DNA radioprotectant for ionizing irradiation. Vitamin C has been shown to significantly reduce damage to gamma irradiated calf thymus DNA at 30-150 Gy[47]. Ascorbate has also been proven to protect against irradiation at 50 kGy, which is considerably closer to terminal dose[48]. High level sterilization of human plasma causes similar chemical modifications of proteins compared to soft tissue, resulting in loss of function. Ascorbate supplementation was able to significantly reduce the amount of irradiation generated protein hydroxyperoxides at 50 kGy[48].

2.5 Riboflavin

Chemical versatility of riboflavin (vitamin B2) is characterized by its many biological functions[66]. Riboflavin often regarded as a contributor to oxidative stress, but also has free radical scavenging ability[66]. This variability is attributed to its participation in metabolic reactions as a result of its ability to transfer electrons[66]. Riboflavin is composed of two subunits ribitol and flavin. Ribitol is a sugar alcohol like mannitol and the flavin subunit possesses an isoalloxazine ring[67]. This ring, which is really a set of 3 rings, can undergo up to two redox reactions similarly to ascorbate[67] (Fig. 2-5, 2-6). Overall, riboflavin has scavenging ability from both its ribitol and isoalloxazine groups[49]. Riboflavin has had a long established role as a biological radioprotectant in cells. Two derivatives of riboflavin in cells flavin mononucleotide (FMN) also known as riboflavin 5'-phosphate, and flavin adenine dinucleotide (FAD), which both have the ability of releasing electrons[49]. Much like the delocalized electrons in vitamin C, the mobility of electrons around this ring contributes to resonance stability. A previous study has shown that nutritional deficiency of riboflavin has been correlated with increased risk of cancer, which is related to the presence of free radicals[49].

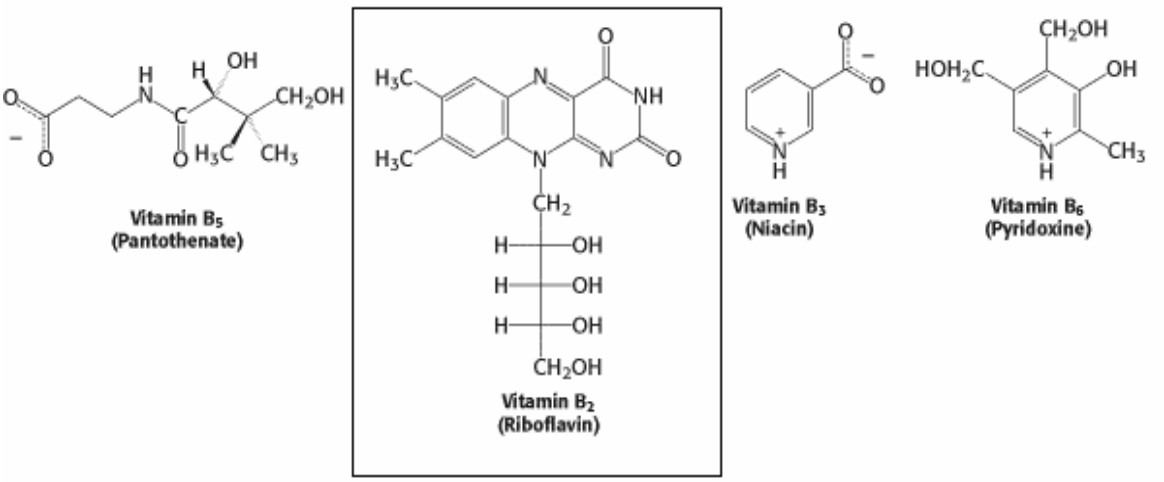


Figure 2-5 Assorted types of riboflavin

We have selected riboflavin (Vitamin B2) for its possible radioprotective properties[65].

Riboflavin has shown radioprotective effects for Escherichia coli cells exposed to gamma irradiation similar to vitamin C[49]. Gamma irradiated bacteria in riboflavin supplemented media exhibited higher populations than control buffered media[49]. As another tribute to riboflavin versatility, photocrosslinking of collagen has also been demonstrated using riboflavin with ultraviolet light (UV)[42,68-70]. Photocrosslinking has been studied as a treatment for keratoconus in cornea[68], and more recently applied to collagen scaffolds[71].The process involves applying riboflavin-adenosine-phosphate to cornea (type II collagen) followed by exposure to UVA irradiation[68]. As a treatment for the degenerative optical disease keratoconus, in vitro crosslinking has shown to increase mechanical strength as well as protease resistance[68,70].

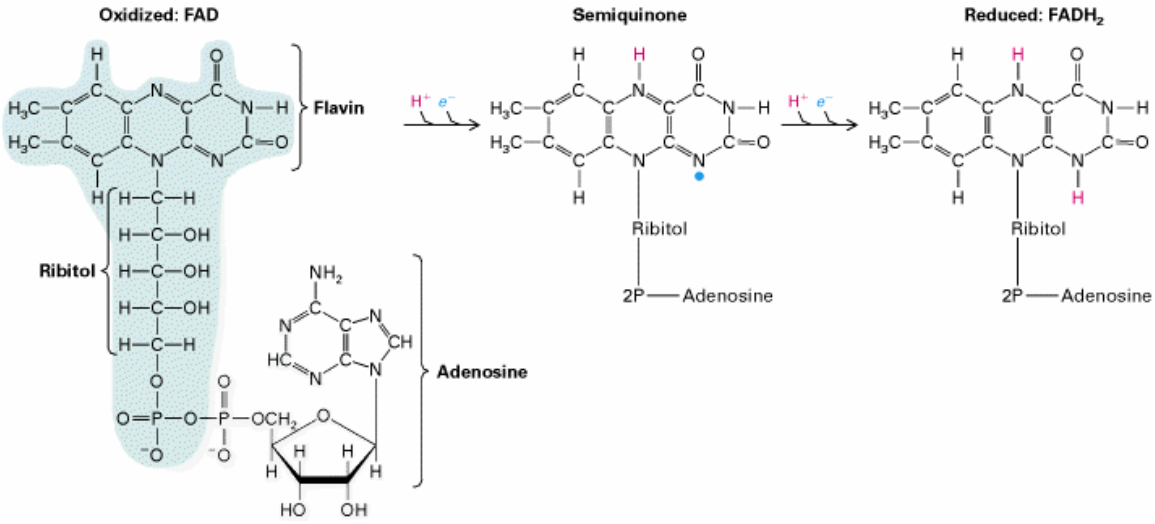


Figure 2-6 Redox capability of riboflavin

Riboflavin a section and precursor of flavin mononucleotide (FMN) highlighted in blue[72]. It has access to three oxidation states and enabling it to conduct two electron transfers in the metabolic cycle[67]. This ability comes from the isoalloxazine triple ring in riboflavin[67]. The presented reaction is the two electron reduction of FAD to $FADH_2$. This is a redox reaction and can also proceed in the reverse.

2.6 Concentration selection

After treatment groups were selected, decisions regarding concentrations also had to be made. This was again difficult due to the lack of literature utilizing these treatments in tissues, as opposed to solution or pure collagen constructs. It was initially thought that high concentrations of free radical scavengers would result in maximal effects. Later concentration effects were studied for glucose and ascorbate, however materials did not permit this for mannitol. Three concentrations were evaluated 500 mM, 100 mM, and 50 mM. Riboflavin was a late addition to treatment groups and optimal concentrations for glucose and ascorbate (Appendix Fig. A-1) were carried over. Selection of EDC concentration was based off of previous lab research regarding crosslinking of collagen fiber bundles for ACL reconstruction[54,58].

3 Methods

3.1 Overview

The motivation behind this research is to investigate crosslinking and free radical scavenging techniques to improve graft properties after sterilization with ionizing irradiation. The study was divided into several phases with individual goals. Phase I was focused on gathering baseline data on the effects of gamma and ebeam irradiation on the rabbit tendon allografts. In this process, freezing and freeze-drying storage methods were also compared. Phase II involved applying crosslinking and free radical scavenging methods, which was followed by irradiation. Effects of irradiation were compared to baseline data to determine success of treatment groups. After Phase II, preliminary work was begun on studying the combination of EDC crosslinking and free radical scavenging in Phase III (Table 3-1).

3.2 Phase I

The purpose for assessing irradiation effects on untreated tendons was to provide comparison to previous studies and for our treatment groups. Additionally provided is a comparison between gamma and ebeam irradiation, as well as frozen and freeze dried (FreeZone freeze dryer Labconco, Kansas City, MO) irradiated conditions. Descriptions of testing methods and preparation are discussed below.

3.2.1 Dissections

Achilles tendons from New Zealand White Rabbits were used as the model for allografts. Frozen rabbit hindlimbs were obtained from Pel-Freeze Bio (Rogers, AR). Achilles tendons were selected because they are relatively large in size for easier handling. Furthermore, human Achilles tendons are commonly used allografts for ligament replacement[3,73-75]. Anatomically, three Achilles tendons are bundled together in rabbits. Tendons were dissected from hindlimbs, wrapped in phosphate buffer solution (PBS) soaked gauze, and placed in 15 ml polystyrene centrifuge tubes. These test tubes stored within a -20 °C freezer. Tendons were assigned to Phase I, Phase II, or Phase III groups.

Table 3-1 Study overview.

This is a brief table displaying where harvested tendons are allocated, as well as sterilization conditions and testing.

<table>
<tr><th>Materials</th><th>Study Breakdown</th><th>Treatments</th><th>Dose Conditions</th><th>Evaluation</th></tr>
<tr><td rowspan="8">Dissections: Harvesting Achilles tendon</td><td>Phase I: Baseline Studies</td><td>Untreated</td><td rowspan="8">25 kGy Gamma Irradiation

25 kGy Ebeam Irradiation

50 kGy Gamma Irradiation

50 kGy Ebeam Irradiation</td><td rowspan="8">Mechanical Testing

Collagenase Resistance

Trypsin Resistance</td></tr>
<tr><td rowspan="4">Phase II: Investigation of Treatments</td><td>EDC</td></tr>
<tr><td>Glucose (Gluc)</td></tr>
<tr><td>Mannitol (MA)</td></tr>
<tr><td>Ascorbate(AS)</td></tr>
<tr><td rowspan="3">Phase III: Investigation of Dual Treatments</td><td>Riboflavin (RB)</td></tr>
<tr><td>EDC/MA</td></tr>
<tr><td>EDC/AS</td></tr>
<tr><td>EDC/RB</td></tr>
</table>

3.2.2 Gamma and Ebeam irradiation

Effects of both gamma and ebeam irradiation were tested for the purpose of comparison. Tendons to be irradiated were packaged on dry ice and sent to irradiation facilities. Gamma irradiation was performed using a Co-60 source at doses 25 kGy and 50 kGy by Sterigenics Inc. (Rockaway, NJ). Ebeam irradiation was performed using a 5 MeV electron accelerator by Ebeam Services Inc. (Cranbury, NJ) at the same doses. 25 kGy was chosen as the IAEA suggested standard dose and as a median of dose ranges currently used by tissue banks[4,5,10], and 50 kGy as closer to a terminal dose for comparison. After return shipping, frozen tendons were returned to the -20 °C freezer and freeze dried tendons left in a vacuumed dessicator until testing. All treated tendons were shipped and irradiated in chilled solution. Tendons not subjected to irradiation were also on dry ice or in chilled solution for equal amount time for shipping and processing.

3.2.3 Mechanical testing

Mechanical properties are an indirect predictor of nativity of the collagen molecule and extent of collagen crosslinking. Lateral covalent crosslinks resist axial load, therefore higher strength corresponds to higher crosslink density[37]. It is also known that damage to crosslinks or loss of nativity results in weakened mechanical properties[13]. Tendons designated for mechanical testing were first thawed out in PBS and left to soak for 30 minutes. Freeze dried tendons were also rehydrated and soaked in PBS for 30 minutes. Dimensional measurements

were taken using a Z-Mike 1202B (Dayton, OH) series laser micrometer. Tendons were mounted against the beam of the laser and held with minimal tension. Two measurements were taken for both thickness and width. Cross-sectional area was estimated by assuming a rectangular shape. Tensile testing was performed on an Instron model 4204 (Canton, MA) testing machine mounted with Enduratec freeze clamps (Eden Prairie, MN). Tendons were mounted in clamps and allowed to freeze until 1mm of the tendons were visibly frozen outside of the clamps. PBS was also regularly applied to maintain hydration. Prior to testing, tendons were first preconditioned in tension at about 1.5 N to 3.5 N for 5 cycles. Gauge length was taken as the distance between frozen ends. Tendons were then pulled in tension at a speed of 100 mm/min until failure to simulate acute trauma. Samples that displayed slippage during testing were excluded from the data pool. Raw data was collected using a Smart Mother Board data collector (Microstrain Inc., Williston, VT). Structural and mechanical parameters were calculated from raw data, and Load Deformation and Stress-Strain curves were constructed using a custom written Matlab program (Norwood, MA) (Appendix F). Both structural and material properties were calculated (Table 3-2), but more emphasis was placed in material properties due to the inconsistency in dimensions of natural tendon. All values are reported as mean ± standard deviation.

Table 3-2 Mechanical parameters calculated

	Parameter	Calculation	Units
Structural Properties	**Break Load**	**Max Force**	**Newtons (N)**
	Stiffness	**ΔForce/ΔLength**	**N/mm**
	Deformation	**Final Length- Gauge Length**	**mm**
	Energy	**Integral of Force v Deformation Curve**	**N*mm**
Material Properties	**UTS (ultimate tensile stress/ strength)**	**Break Load/ Cross section Area**	**Mega Pascal (MPa)**
	Elastic Modulus	**ΔStress/ΔStrain**	**MPa**
	Strain	**Deformation/Gauge**	**None**
	Toughness	**Integral of Stress v Strain Curve**	**MPa**

3.2.4 Collagenase resistance test (CRT)

Collagenase resistance testing is a qualitative measurement related to the in vivo resorption rate of implanted collagen materials[76]. Collagenase is among natural enzymes released during the wound healing process to break down proteins[77]. Similarly to mechanical testing, it is also an indirect measure of extent of crosslinking. Uncrosslinked collagen is more susceptible to collagenase breakdown than if it were crosslinked[38]. Bacterial collagenase derived from clostridium histolyticum was used for this study and cleaves the bond between the 'X' and glycine amino acids in backbone –X-glycine-proline-Y- sequences[78].

Bacterial collagenase was chosen for its wider range of cleavage sites than Mammalian collagenase[42], and as a result differences in dissolution time were more discernable.

Tendons were first lyophilized to obtain accurate dry weight and then sectioned into 3.5 mg segments. These segments were placed in test tubes and soaked in 10 ml collagenase solution (400 units/ml). This solution was made using 1 M Tris buffer, deionized water, and clostridiopeptidase A (Sigma-Aldrich St. Louis, MO), derived from clostridium histolyticum. A CRT was performed for each group in triplicate with collagen sponges included as positive controls. Test tubes were placed in a 37 °C water bath and observed every half hour for a total of 24 hours. At each time point, a score was recorded based on visible degradation with a 5 signifying intactness and 0 completely disintegrated. However the most important parameter was average dissolution time between initial soak and disintegration.

3.2.5 Trypsin resistance test (TRT)

A trypsin resistance test, like collagenase, is a method of qualitiatively assessing the survivability of an implanted material in the in vivo environment. Also like collagenase, trypsin is a method to determine crosslink density because susceptibility will be reduced when crosslinks restrict access to cleavage sites. Trypsin cleavage sites are more selective than collagenase, by only cleaving between lysine and arginine residues in denatured collagen[78]. Trypsin susceptibility can also be used as a measure of the amount of denaturation of

collagen. Non-denatured collagen is very resistant to trypsin digestion. Collagen digestion by trypsin is resisted by two factors: 1) penetrability into the network structure of tissue and 2) steric hindrance by covalent intermolecular crosslinks blocking active sites[79]. For our purposes we are seeking to determine the amount of surviving crosslinks after irradiation. Therefore in order to allow trypsin penetration, tendon samples have to be denatured. Heat denaturation will only open hydrogen bonding in collagen, and should have no effect on crosslinks.

Trypsin resistance was only performed on groups necessary to complete Phase II and Phase III experiments, due to a limitation in materials. Preparation for trypsin resistance is similar to CRT. First whole tendons were lyophilized to obtain dry weight, which were on average of 30 mg. 1000 unit/ml trypsin solution was prepared using trypsin (Sigma-Aldrich St. Louis, MO) and PBS. Trypsin resistance was performed in triplicate as in CRT, again with collagen sponges were also used as a positive control. Lyophilized tendons were then placed in boiling water at 100 °C for 2 minutes to denature the collagen. They were then placed in test tubes filled with 10 ml of trypsin solution, and then placed in a hot water bath at 37 °C for 48 hours. Tendons that remained after 48 hours were removed and lyophilized again to obtain dry weight after trypsin digestion. Percent digested by trypsin was reported ± standard deviation for each group. The amount of weight loss is indicative of the amount of existing crosslinks and nativity of collagen.

3.3 Phase II

3.3.1 EDC crosslinking

Tendons were crosslinked 2 per 15 ml centrifuge tube in a solution of 10 mM EDC supplemented with 5 mM NHS (N-hydroxyl succinimide) in deionized water (Sigma-Aldrich St. Louis, MO). Tendons were set in solution for 24 hours and then washed 3 times in deionized water in 10 minute intervals. Then tendons were soaked in .1M $NaHPO_4$ (Sigma-Aldrich St. Louis, MO) and deionized water solution for 2 hours. Tendons were next rinsed in deionized water for another 24 hours to complete the process. Tendons were stored at 4 °C during crosslinking and washing steps. A total of 32 tendons were crosslinked for this group, with 8 tendons per dose condition. Tendons were subjected to 25 kGy and 50 kGy of gamma and ebeam irradiation. These figures were identical over each treatment condition.

3.3.2 Glucose crosslinking

As mentioned, glucose crosslinking in collagen naturally occurs through the Maillard reaction [55,56], but known to be expedited in the presence of free radicals[60,61]. Therefore in this expedited procedure, the majority of glucose crosslinking occurs at the time of irradiation. For this to occur, glucose must be present in solution with the tendon during irradiation. Preparation involved presoaking in a 100 mM solution of D (+) glucose, 99.5% anhydrous (Sigma-

Aldrich St. Louis, MO) and PBS for 36 hours in 4 °C. Tendons were soaked 2 per 15ml and irradiated in similar fashion (8 tendons per dose condition).

3.3.3 Free radical scavengers

Preparation of solutions was similar for the 3 scavengers that were used: D-mannitol (Sigma-Aldrich St. Louis, MO), (+) sodium ascorbate (Sigma-Aldrich St. Louis, MO), and riboflavin (vitamin B2) (Sigma-Aldrich St. Louis, MO). Two tendons per 15 ml were soaked in 500 mM mannitol solution, 100 mM ascorbate solution, and 100 mM riboflavin solution in PBS for 36 hours in solution at 4 °C. A total of 32 tendons were used for each group (8 per dose condition) and subjected to 25kGy and 50 kGy of gamma and ebeam irradiation.

3.4 Phase III

3.4.1 Combination of crosslinking and scavenging

Ten tendons per group were first EDC crosslinked following the mentioned protocol. This was followed by soaking in free radical scavenger solutions for 36 hours at the same concentrations as previous protocols. This was made possible because tendons could be pre-crosslinked with EDC, while action of free radical scavengers occurs during irradiation. Glucose crosslinking was not tested in combination groups for several reasons. Both glucose crosslinking and free radical scavenging take action at the time of irradiation, which would result in confounding effects. It was also shown in previous studies that aminoguanidine (a free radical scavenger) inhibits the formation of glucose crosslinks[61]. For this

part of the study, EDC crosslinking was coupled with mannitol (EDC/MA), riboflavin (EDC/RB), and ascorbate (EDC/AS) soaking. These conditions were studied at a dose of 50 kGy.

3.5 Statistical Analysis

Statistical evaluation was performed using SigmaStat analysis software. Student-neuman-keuls (SNK) two-way analysis of variance was performed for mechanical testing data as well as CRT data. The two factors included irradiation (type and dose) and treatment (or untreated). There was specific interest placed on untreated versus treated groups for each dose condition. Individual SNK tests were conducted to determine significance of freeze drying and treatment compared to baseline groups. Analysis for Phase III was isolated to all irradiated tendons at 50 kGy due to the limited dose condition. Significance was noted for P values below .05.

4 Results

4.1 Mechanical Analysis

4.1.1 Phase I Baseline

Baseline studies were conducted on untreated tendons to observe the sole effects of irradiation, as well as a comparison of gamma and ebeam. There was a clear dose-related decline in strength and toughness for both types of irradiation (Fig. 4-1, 4-2). There was a decrease in elastic modulus with the presence of irradiation but dose effects were not as clear. The least affected parameter was strain. Dose effects were most evident in strength graphs, more specifically an inverse relationship between strength and dose. Although these initial experiments were only to study irradiation effects, these trends were generally observed over all sterilization studies. For gamma and ebeam irradiation there was an average of 36% and 55% loss in tensile strength at 25 kGy and 50 kGy compared to unsterilized (Fig. 4-1). Additionally there was a 24% and 67% loss in toughness at these respective doses (Fig. 4-2).

When comparing the effects of irradiation on frozen and freeze dried tendons, it is evident that the effects are similar for freeze dried conditions but to a much higher degree. The effect of freeze drying alone was insignificant; however in combination with irradiation decline was drastic. About a 90% reduction in tensile strength was observed with freeze dried tendons irradiated at 50 kGy for both types of irradiation, compared to frozen irradiated (Fig. 4-1).

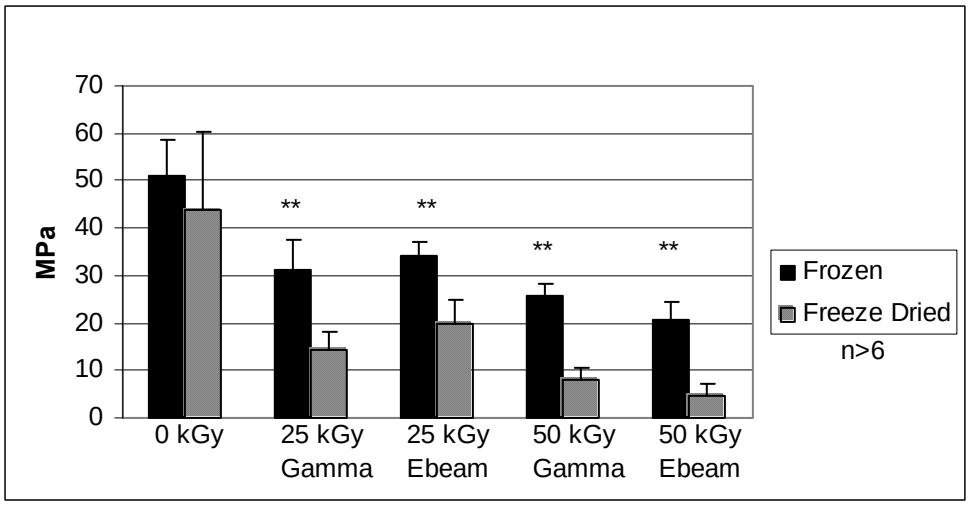


Figure 4-1 UTS of frozen versus freeze dried irradiated tendons.

Freeze drying followed by irradiation is clearly deleterious to tensile strength compared to freezing. At all doses of irradiation freeze dried tendons were significantly weaker than frozen. Effect of freeze drying alone was not significant. Finally, there is a trend in reduction according to dose among untreated groups, which confirm other dose dependent reports. Student-neuman-keuls post hoc analysis. Significant compared to untreated ** (P<.05)

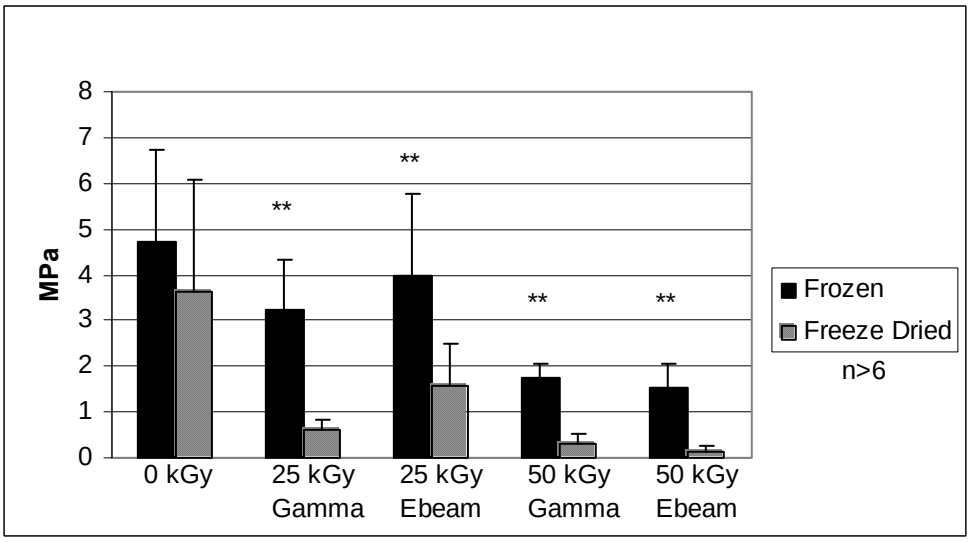


Figure 4-2 Toughness of frozen versus freeze dried irradiated tendons.

There is a clear drop in toughness from increasing from 25 kGy to 50 kGy. The drastic decrease in strength seen with the freeze dried condition is similar for toughness. Additionally, all frozen irradiated conditions have significantly greater

toughness than freeze dried irradiated. Significant compared to untreated ** (P<.05)

4.1.2 Phase II Treatments

4.1.2.1 EDC

EDC crosslinked tendons displayed the highest ultimate stress at 50 kGy for gamma and ebeam irradiation, but not significantly. This represented a 54% and 40% increase compared to untreated tendons at these doses (Fig. 4-3). Elastic moduli were greater than untreated tendons for all doses, and toughness was higher at 50 kGy (Fig. 4-5). However this treatment was not successful in completely restoring mechanical properties to levels comparable to native unsterilized tendon. The presence of dose dependence with EDC treated tendons was difficult to observe in any parameters. Finally, EDC crosslinking alone did not have much of an effect on mechanics compared to untreated tendon.

4.1.2.2 Glucose

The effect of glucose alone was not significant, but both tensile stress and modulus increased when 25 kGy gamma irradiation was applied (Fig. 4-3). The effects of glucose crosslinking were best seen at 25 kGy. Ultimate stress was highest among all other treatment groups irradiated at this dose, and these values were also significant compared untreated. At this dose there was a 64% increase for gamma irradiation, and 45% for ebeam irradiation. These effects

were also mirrored in toughness graphs. At 50 kGy, stress values decreased but still remained higher than untreated. Elastic modulus was significantly higher for all doses, and similarly to EDC crosslinking dose effects were not evident (Fig. 4-5).

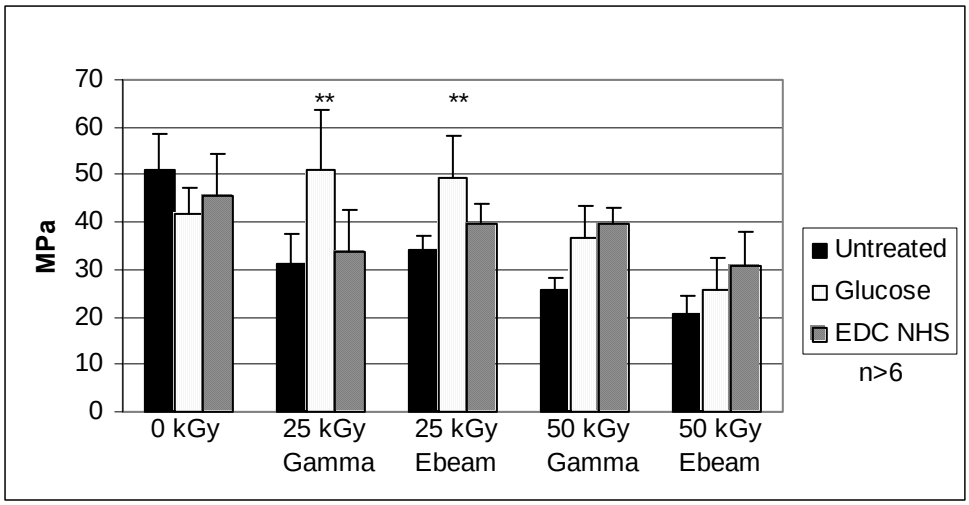


Figure 4-3 UTS of crosslinked irradiated tendons tendons.

Glucose crosslinked tendons have significantly higher strength at 25 kGy than untreated tendons, and are similar to untreated unsterilized tendons. EDC crosslinked tendons had the highest tensile strength among all treated groups at 50 kGy, and were similar to untreated at 25 kGy. Significant compared to untreated ** (P<.05)

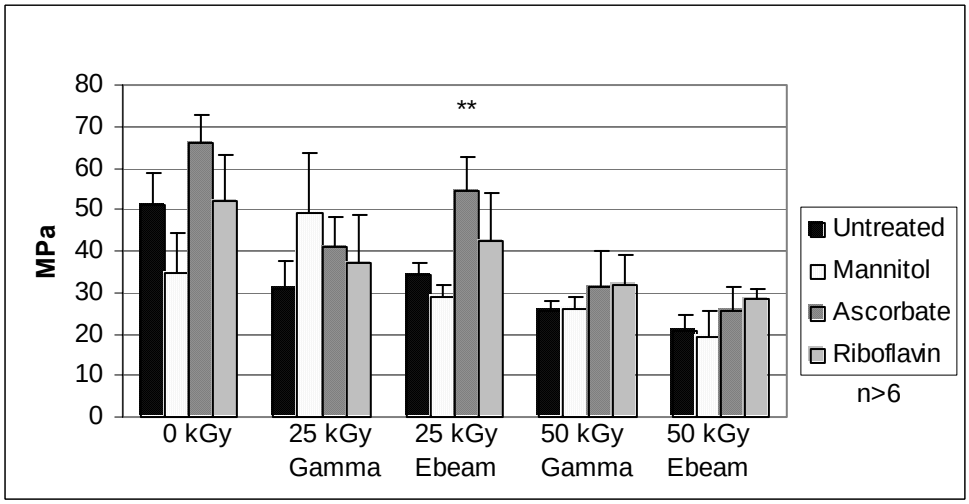


Figure 4-4 UTS of free radical scavenged irradiated tendons.

Radioprotective potential of free radical scavengers was assessed in terms of mechanical properties, and effects were clearly evident with tensile strength. Ascorbate and riboflavin soaked tendons showed selective improvement in UTS. Free radical scavengers were mostly effective at 25 kGy, and effects were diminished at 50 kGy. Significant compared to untreated ** (P<.05)

4.1.2.3 Mannitol

Mannitol was the least effective among treatment groups in terms of improvements compared to baseline data. Mannitol treated groups showed no consistent sign of improvement mechanically aside for a few isolated conditions. At 25 kGy of gamma irradiation mannitol soaked tendons possessed the highest ultimate stress among free radical scavengers, but beyond this dose there was little benefit (Fig. 4-6). At the same time there generally seemed to be no negative effects of mannitol with irradiation, ultimate stress and modulus were close to identical compared to the untreated group. The only decline in mechanical data was in the unsterilized condition (Fig. 4-6).

4.1.2.4 Ascorbate

Ascorbate soaked tendons displayed increased stress and modulus over the whole dose range compared to untreated. Changes in toughness and strain were less consistent. Elastic moduli at 25 kGy, as well as stress at 25 kGy ebeam irradiation were significant compared to untreated (Fig.4-6,4-4). Increases in stress and modulus were best seen at 25 kGy, whereas at 50 kGy effects were more diminished to the levels of untreated. There was still a general trend in reduction with increased dose for ultimate stress and toughness (Fig.4-4,4-8). The dose-related response was evident but less noticeable for modulus values. Ascobate soaking also displayed a higher stress than native tendon without exposure to irradiation.

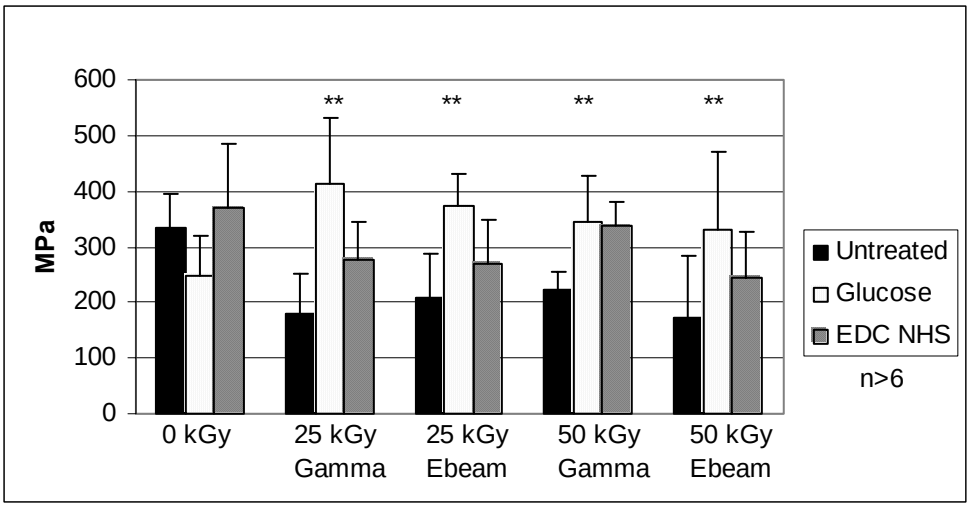


Figure 4-5 Elastic modulus of crosslinked irradiated tendons tendons.

Glucose crosslinked tendons had significantly higher moduli than untreated tendons over the entire dose range. This data supports the findings that the glycation reaction is free radical driven. Modulus and UTS are higher when free radicals are present compared to unirradiated. This evidence suggests crosslinks are being formed in the presence of irradiation derived free radicals. Finally dose effects of irradiation were not as clear in modulus values. Significant compared to untreated ** (P<.05)

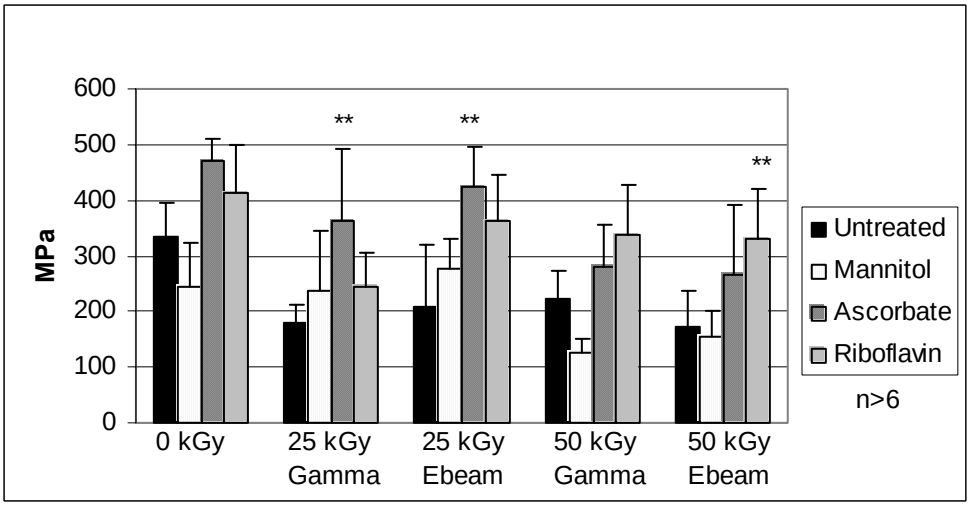


Figure 4-6 Elastic modulus of free radical scavenged irradiated tendons.

Ascorbate and riboflavin soaked tendons had higher modulus values than untreated tendons over the dose range. Ascorbate had significantly higher moduli at 25 kGy. Significant compared to untreated ** (P<.05)

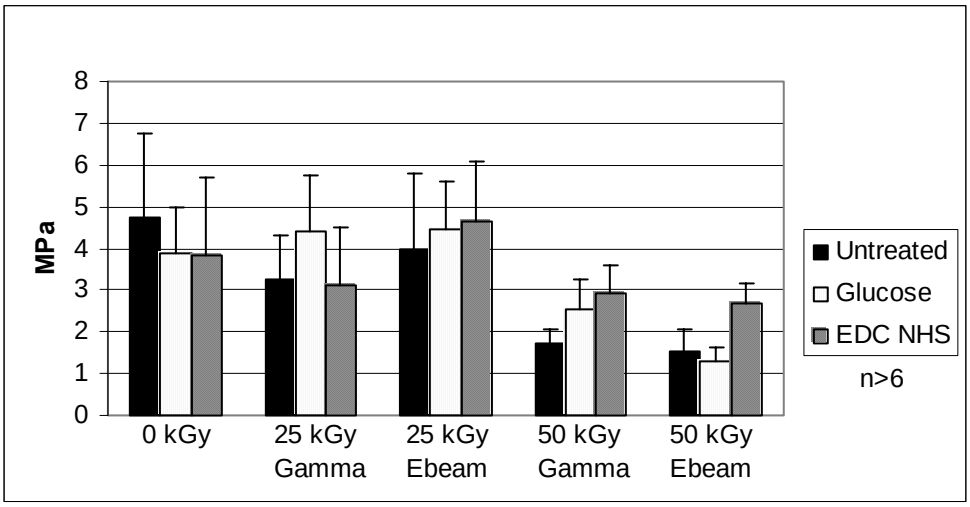


Figure 4-7 Toughness of crosslinked irradiated tendons tendons.

Dose dependent effets were evident for toughness values. At 50 kGy EDC crosslinking is most resistant to irradiation. Crosslinkers possessed toughness close to native tendon for the majority of 25 kGy conditions.

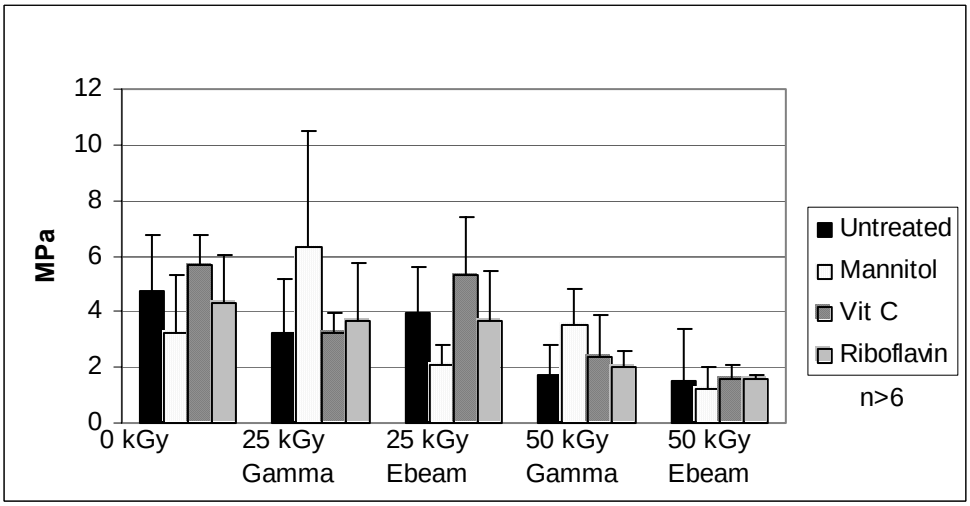


Figure 4-8 Toughness of free radical scavenged irradiated tendons.

Free radical scavengers were for the most part unsuccessful at improving toughness values beyond 25 kGy. As seen in stress values, free radical scavengers are more effective at 25 kGy than at 50 kGy. Additionally, dose related effects are observed in control untreated tendons.

4.1.2.5 Riboflavin

Similarly to ascorbate, riboflavin soaked tendons had both higher stress and modulus than untreated over the entire dose range (Fig.4-4,4-6). Riboflavin effects on stress and modulus were also more prominent at 25 kGy, and diminished at 50 kGy. Differences in strain and toughness were not observable compared untreated. Increase in elastic modulus was significant for 50 kGy ebeam irradiation (Fig.4-6). There was a 23% and 38% increase in stress compared to untreated at 50 kGy gamma and ebeam irradiation (Fig. 4-4). Stress and modulus were highest compared to other scavengers at 50 kGy. Much like other groups, elastic modulus recorded at 25 kGy remained constant with 50 kGy of irradiation.

4.1.3 Phase III Dual Treatments

Preliminary studies were conducted to assess the potential for combination of free radical scavenging and crosslinking. This section of the study was only tested at 50 kGy. The effects of dual treatment prior to irradiation are similar to EDC treatment. Crosslinking resulted in an increase in modulus, and possibly brittleness which is observed with decreased toughness. Soaking in free radical scavenger solution had little effect on unirradiated tendons (Fig. 4-9).

Dual treated groups were the best protectors of mechanical properties after irradiation. All combined treatments showed better strength and modulus than any single treatment alone at 50 kGy. Similarly to other irradiated conditions, there was no difference between gamma and ebeam irradiation.

Furthermore statistical analysis declared all these values to be significantly greater than untreated except the elastic modulus for EDC/RB. For EDC crosslinking with mannitol, riboflavin, and ascorbate soaking, average strength increases for gamma and ebeam irradiation were 93%, 89%, and 107% compared to untreated (Fig. 4-10). Toughness was also higher than untreated but not to the level of native tendon.

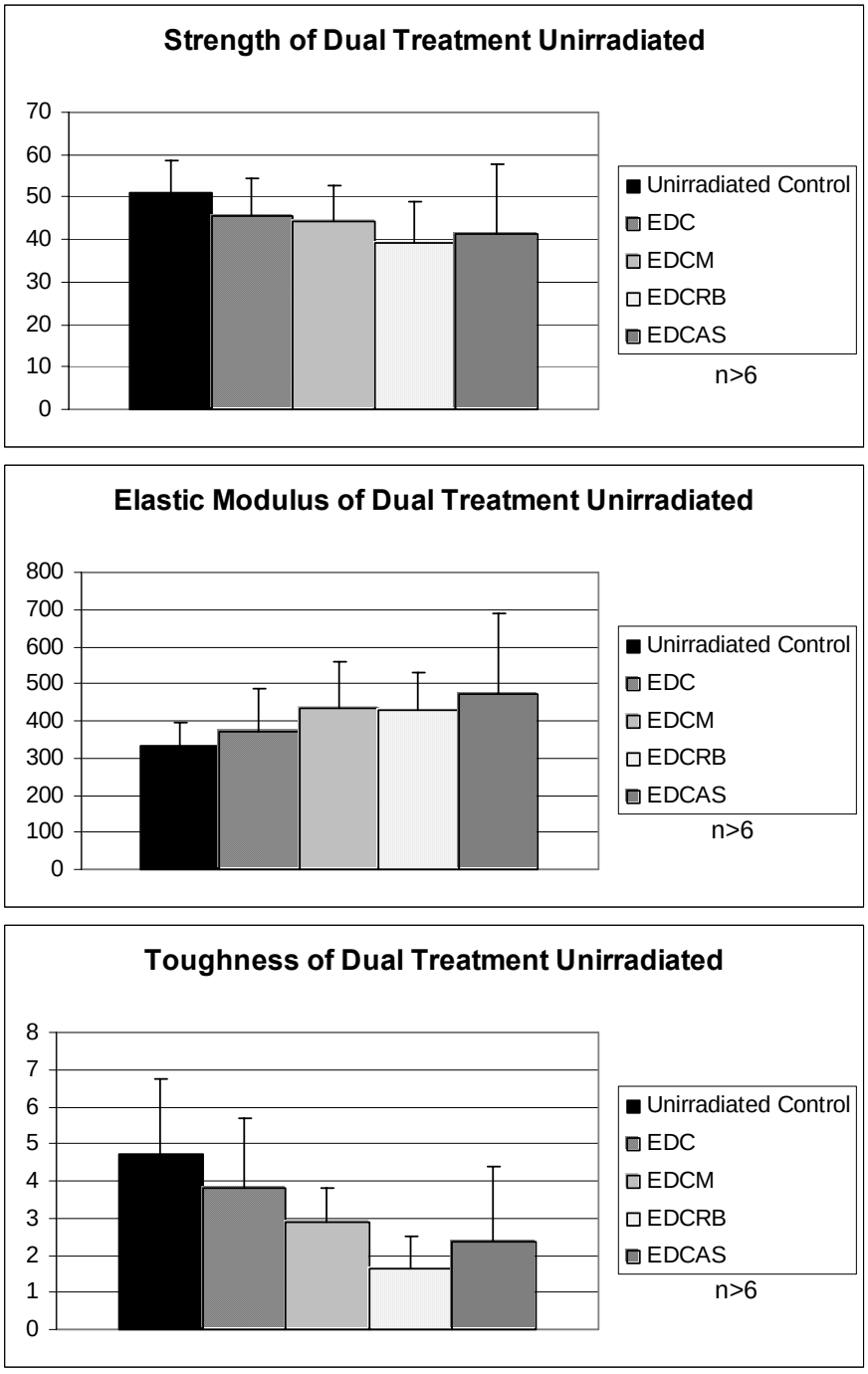


Figure 4-9 Mechanical properties of dual treated tendons unsterilized.

Strength, Elastic Modulus, and Toughness of dual treated tendons. There was not much of a mechanical effect of treatment alone. There was a slight increase in elastic modulus with treatments involving EDC crosslinking.

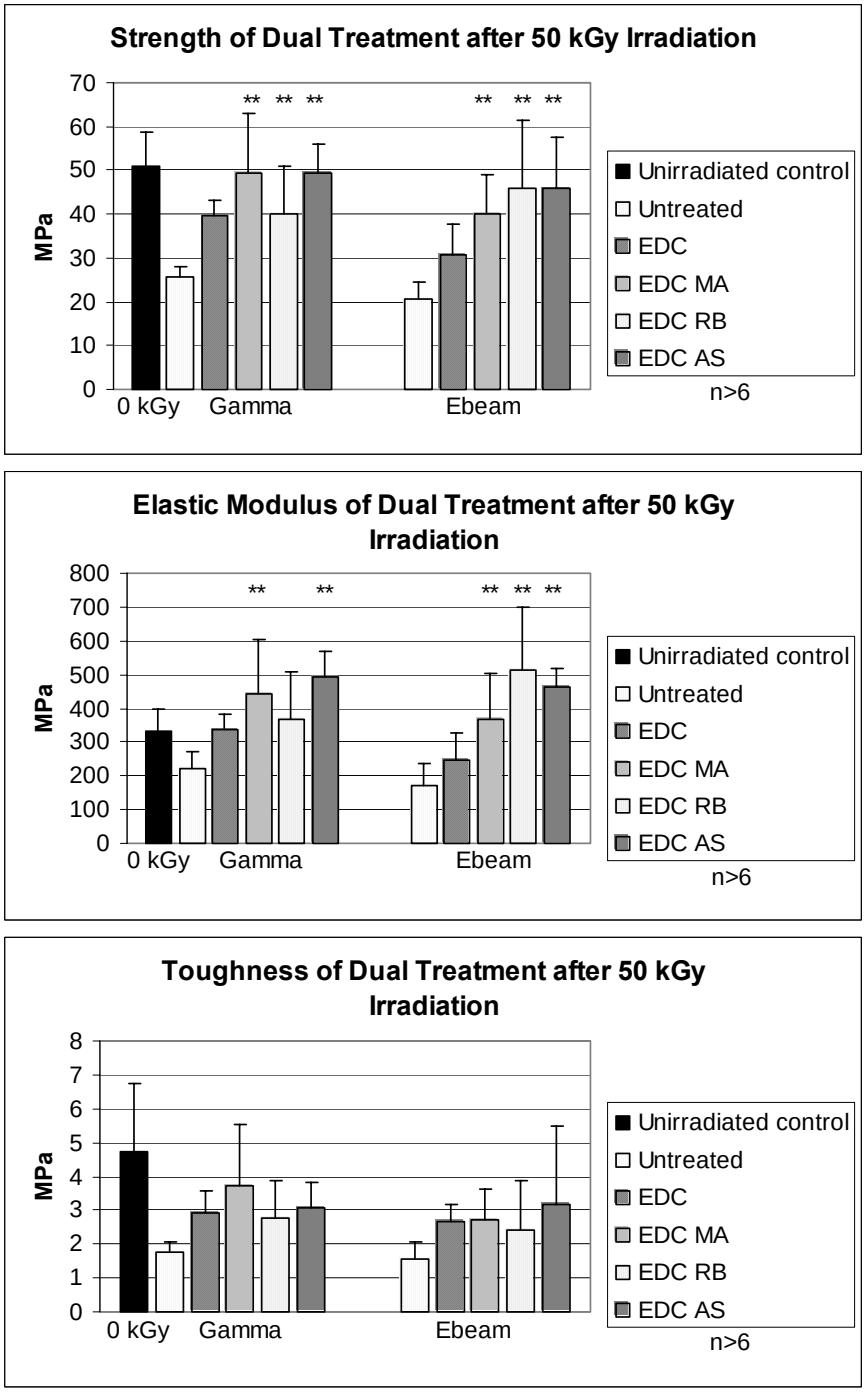


Figure 4-10 Mechanical properties of dual treated tendons at 50 kGy.

All combinations of EDC crosslinking and scavenging showed protective effects and possessed strength and modulus values comparable to native tendon. Mechanical properties of combined groups were also higher than that of individual treatments alone. Significant compared to untreated ** (P<.05)

4.2 Collagenase Resistance

Baseline groups

An effect by ionizing irradiation on collagenase resistance was identified, although it did not seem to have the sensitivity to distinguish differences between 25 kGy and 50 kGy doses (Fig. 4-11). Resistance was slightly higher for the 25 kGy dose compared to 50 kGy. This observation was also seen with treatment groups. Among baseline results, there was a clear decrease in resistance when irradiation was applied. It was also difficult to see a difference between frozen and freeze dried conditions, but frozen tendons did have slightly higher resistance compared to freeze dried. Meanwhile freeze drying and irradiation resulted in drastic effects after mechanical testing. Consequently, emphasis was placed on comparisons that showed significant differences.

Treatment groups

EDC crosslinking was by far the most resistant to collagenase, and remained intact for the majority of the dose conditions for the entire 24 hour study (Fig. 4-12). Only at 50 kGy of irradiation did these tendons dissolve prior to 24 hours. Additionally, both glucose and riboflavin showed a significant increase in resistance for ebeam irradiation of 25 kGy and 50 kGy compared to untreated (Fig. 4-12, 4-13). Although the majority of other groups showed increases in resistance compared to untreated for individual dose conditions, they were only slight and not considered significant.

Dual Treatment groups

Collagenase resistance of dual treatments exposed to 50 kGy, was highest among all treatment groups. There was slight degradation but they remained intact for 24 hours in collagenase solution for both irradiation types. Dual treatments were more resistant than EDC crosslinking alone, which degraded after 18 and 22 hours at 50 kGy gamma and ebeam irradiation. Although this study was conducted for a maximum 24 hours, it is likely that dual treated groups would resist collagenase degradation far beyond this point.

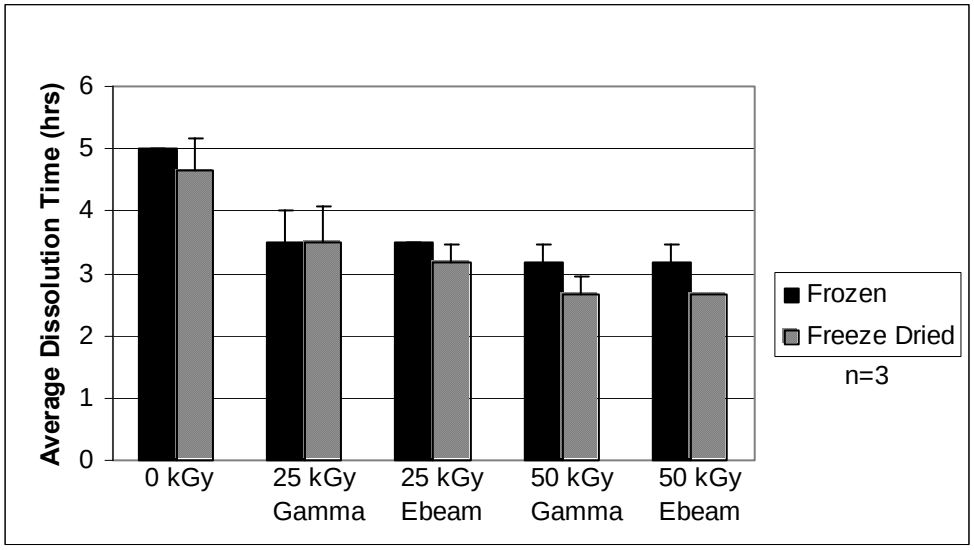


Figure 4-11 CRT of frozen versus freeze dried irradiated tendons.

The influence of irradiation on dissolution time is evident, however the dose response observed in mechanical properties is not distinguishable in collagenase resistance. Difference between frozen and freeze dried conditions is also not evident as observed in mechanical data.

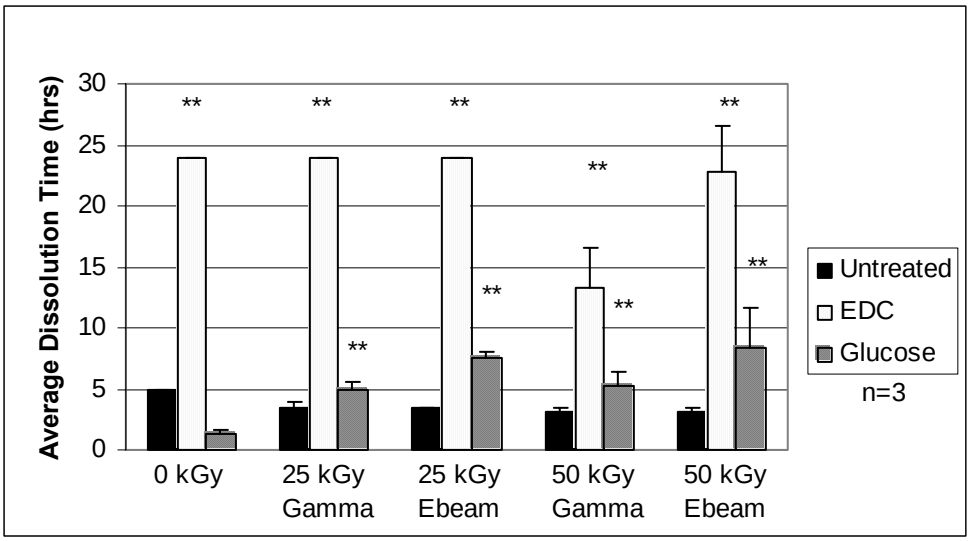


Figure 4-12 CRT crosslinked irradiated tendons.

Enzyme resistance testing showed that EDC is clearly the most resistant to collagenase, remaining intact at most doses for the 24 hr study. Glucose also showed significant increases in resistance compared to untreated for ebeam irradiation at 25 kGy and 50 kGy. Significant compared to untreated ** (P<.001)

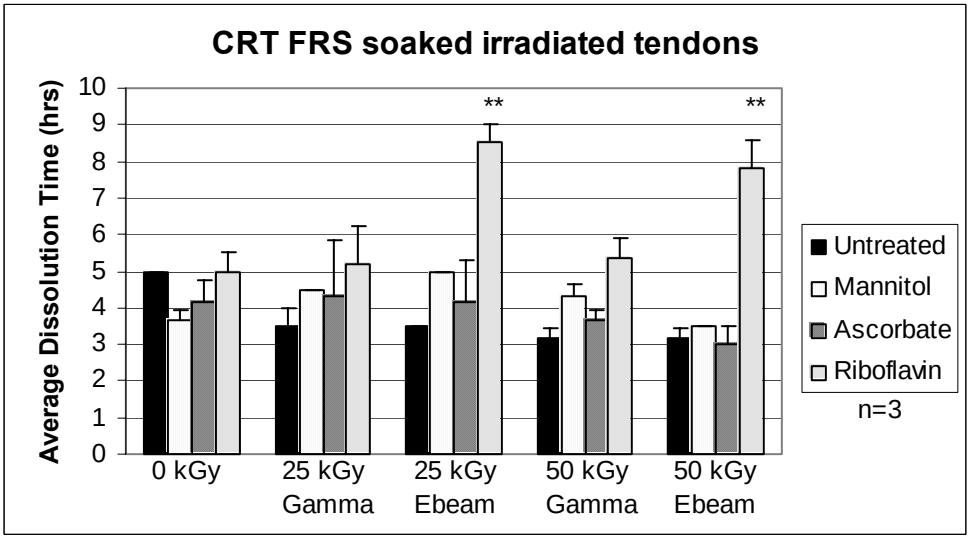


Figure 4-13 CRT free radical scavenged irradiated tendons.

Differences between 25 kGy and 50 kGy of untreated tendons were slight. There were no clear effects of free radical scavengers in terms of collagenase resistance, except for the riboflavin condition. Riboflavin also showed significant increases in collagenase resistance for ebeam irradiation at 25 k Gy and 50 kGy. Significant compared to untreated ** (P<.001)

4.3 Trypsin resistance

Effect of irradiation was also evident after boiling and exposure to trypsin solution. Trypsin resistance is a measure of degree of denaturation of collagen as well as a measure of crosslinking[78]. Upon visual observation tendons remains exposed to 25 kGy maintained more of a continuous matrix structure, whereas tendons remains exposed to 50 kGy were more particulate. Dose conditions displayed the most obvious effects on trypsin resistance. As more irradiation was applied the amount of weight loss after trypsin exposure increased. Digestion of tendons exposed to 50 kGy irradiation was greatly accelerated. These tendons were completely dissolved after 4 hrs in trypsin solution for gamma irradiation. Additionally, tendons gamma and ebeam irradiated at 25 kGy did not completely dissolve but displayed weight loss at 84% and 92% (Table 4-1). None of the treatment groups dissolved at any dose condition.

There were slight differences between treatment conditions, similary to collagenase. As for dose conditions, there were slightly increases in resistance for the lower dose. Protective effects of treatment were more evident for gamma irradiated tendons. Differences between treatments were more difficult to distinguish for ebeam irradiated tendons, and in general were more clear with gamma irradiated tendons. The potential reasons are discussed later in section 5. Briefly focusing on gram irradiated tendons, crosslinkers glucose and EDC possessed highest resistance to trypsin degradation, which was also similar to collagenase testing. Compared to unirradiated glucose treated tendons retained

more weight as dose increased. Ascorbate and riboflavin treated tendons also displayed this trend but weight retention was slight.

Table 4-1 Trypsin Digestion: Phase II and Phase III groups

Data is reported as average percent and standard deviation

Unirradiated	Percent Weight Loss		Gamma Irradiated	Percent Weight Loss
Untreated	75.5% (8.8)		Untreated (25 kGy)	83.9% (12.8)
			Untreated (50 kGy)	100% (0)
EDC	68.2% (15.5)		EDC (25 kGy)	56.3% (13.4)
			EDC (50 kGy)	65.7% (12.7)
Glucose	70.0% (13.4)		Glucose (25 kGy)	49.4% (15.8)
			Glucose (50 kGy)	55.7% (31.0)
Mannitol	60.2% (17.5)		Mannitol (25 kGy)	83.8% (12.7)
			Mannitol (50 kGy)	68.9% (21.8)
Ascorbate	68.0% (22.2)		Ascorbate (25 kGy)	60.5% (12.0)
			Ascorbate (50 kGy)	59.7% (32.8)
Riboflavin	76.6% (16.7)		Riboflavin (25 kGy)	74.0% (18.3)
			Riboflavin (50 kGy)	70.3% (31.4)

Unirradiated	Percent Weight Loss		Ebeam Irradiated	Percent Weight Loss
Untreated	75.5% (8.8)		Untreated (25 kGy)	92.4% (2.87)
			Untreated (50 kGy)	97.1% (.358)
EDC	68.2% (15.5)		EDC (25 kGy)	81.9% (9.43)
			EDC (50 kGy)	95.7% (3.51)
Glucose	70.0% (13.4)		Glucose (25 kGy)	94.8% (3.01)
			Glucose (50 kGy)	96.6% (1.53)
Mannitol	60.2% (17.5)		Mannitol (25 kGy)	84.3% (8.20)
			Mannitol (50 kGy)	96.5% (1.28)
Ascorbate	68.0% (22.2)		Ascorbate (25 kGy)	91.1% (3.24)
			Ascorbate (50 kGy)	92.7% (4.56)
Riboflavin	76.6% (16.7)		Riboflavin (25 kGy)	89.3% (2.61)
			Riboflavin (50 kGy)	97.9% (.321)

Dual Treatments

Unirradiated	Percent Weight Loss		Gamma Irradiated	Percent Weight Loss
Untreated	75.5% (8.8)		Untreated (50 kGy)	100% (0)
EDC M	65.3%(10.5)		EDC M (50 kGy)	68.2%(22.2)
EDC AS	70.3%(12.2)		EDC AS (50 kGy)	77.3%(6.5)
EDC RB	69.5%(6.6)		EDC RB (50 kGy)	71.2%(20.2)

Unirradiated	Percent Weight Loss		Ebeam Irradiated	Percent Weight Loss
Untreated	75.5% (8.8)		Untreated (50 kGy)	97.1% (.358)
EDC M	65.3%(10.5)		EDC M (50 kGy)	94.9% (1.54)
EDC AS	70.3%(12.2)		EDC AS (50 kGy)	90.0% (2.81)
EDC RB	69.5%(6.6)		EDC RB (50 kGy)	85.0% (7.90)

5 Discussion

This research was aimed at stabilizing allografts, specifically improving mechanical response and enzymatic resistance, which are compromised after exposure to ionizing irradiation. The main consequence of ionizing irradiation is modification of collagen by free radicals. Collagen content, nativity, and crosslinks are determinants of mechanical stability and enzyme degradation of allografts[38,76]. Having identified the obstacle, the two distinct methods chosen to address these effects were crosslinking and free radical scavenging. These approaches are specifically aimed to maintain collagen nativity and crosslinking after irradiation. It was hypothesized that these methods would protect mechanical properties and resistance to degradation allowing the use of ionizing irradiation for terminal sterilization. Also recall the intent of this research was to test a large pool of protective treatments and identify those that were successful.

The majority of treatment groups demonstrated success in improving mechanical properties and collagenase resistance. Among treatment groups, there was no single treatment that displayed overwhelming benefit over other groups. However, crosslinking groups did provide slightly more consistent improvement compared to free radicals scavengers. Treatments were unsuccessful in completely reversing irradiation damage, but there were significant improvements. Introductory studies investigating combination of crosslinking and scavenging have produced even better results. Although this research places emphasis on the performance of crosslinking and scavenging

treatments, it also provides insight regarding comparison between gamma and ebeam irradiation on tendons as well as assessment of freeze drying followed by irradiation. These studies serve as a basis for comparison, but also contribute knowledge that has not yet been previously presented.

5.1 Phase I Baseline

5.1.1 Effect of gamma and ebeam irradiation

Deleterious effects on mechanical properties are a pressing obstacle against the use of ionizing irradiation for terminal sterilization[10]. Dose related effects of gamma irradiation on collagen-based materials have been well established in the literature[10,13,14]. This relationship between dose and decrease in mechanical properties has been found to be inverse and nonlinear[15]. Our data reports substantial decreases in strength and toughness, and an evident but less dose responsive effect on elastic modulus. This trend was observed globally for all irradiated samples. Perhaps more accurate description of dose effects requires focus on stress and toughness. Although there are certain structural differences between tendon and bone, it is perhaps noteworthy to report that these effects were observed in gamma irradiated bone allografts[12,15]. There is an agreement among bone studies that irradiation most affects plastic properties of bone namely ultimate strength and toughness, with less differentiable influence on modulus and strain[12,15]. Strength and toughness are parameters that are

directly proportional to break load, which is known to be dependent on collagen crosslinking. Conversely deformation seems to be less effected.

Dose dependent effects among untreated tendons were more discernable in trypsin resistance compared to collagenase. There was a clear trend in weight loss after trypsin digestion, increasing with dose as seen in Table 4-1. However for collagenase resistance, time to dissolution for increasing dose was very close. Tendons irradiated at 25 kGy were slightly more resistant than at 50 kGy, implicating less damage caused by irradiation which supports trypsin and mechanical data. The reason differences were not as pronounced as other tests, which could be due to the fact that such a high concentration of collagenase was used. Briefly, a high concentration results in accelerated breakdown that may be too difficult to record. However, fast degradation would mean small differences in time are more significant. This discussion is continued further in section 5.4.

Decreases in strength, toughness, and modulus are evident during irradiation at the lower dose of 25 kGy and are more pronounced at 50 kGy. It has been suggested in the past that 25 kGy to be an unofficial standard dose, before mechanical properties are affected[19]. Despite the fact that free radical migration is impeded in frozen materials and weakening is already minimized, there were decreases in mechanical properties at 25 kGy. It is possible that differences in our data could be the result of different environmental conditions. Based on these results, it is not recommended that this dose be used at these processing conditions. Finally, direct comparison of gamma and ebeam irradiation shows effects on untreated tendons was nearly identical. This

knowledge potentially leaves more options available for sterilization of collagen allografts.

5.1.2 Combination of freeze drying and irradiation

Freeze dried conditions were studied to compare effect of water content on irradiation. Mixed results have been reported among studies assessing the combination of freeze drying and ionizing irradiation. Our results reaffirm the majority of other groups that show drastic decline in material properties beyond that seen with frozen conditions[80-83]. Some have found no difference in mechanical properties between frozen and freeze dried irradiated tendon allografts[8,9]. However recall accurate comparison can only be made if processing conditions are the same, eliminating environmental factors[11]. Explanations of declined strength, which declare that free radicals contribute to crosslink formation are under debate[81]. These studies claim that by reducing water content, the amount of oxygen derived free radicals is decreased, thereby decreasing crosslink formation[19,80,81]. Others agree that free radicals participate in crosslinking to a degree, but primarily cause breakdown of crosslinks and are the main cause of damage by ionizing irradiation[11]. There have been no further studies to substantiate the theory, so the question has yet to be resolved. Although it is clear that these conditions, collagen materials in freeze dried storage should not be irradiated if mechanical integrity is desired.

5.2 Phase II Treatments

5.2.1 EDC

Although EDC crosslinking has found expanding use for collagen materials, the lack of literature describing effects in the presence of ionizing irradiation does not allow for much comparison. EDC crosslinked tendons were resistant to dose effects of irradiation, meaning there were few differences between tendons irradiated at 25 kGy and 50 kGy. Among phase II treatment groups, EDC exhibited the highest mechanical strength at 50 kGy, though not significantly. This strength was comparable to untreated tendons irradiated at 25 kGy. This group also showed the highest resistance to collagenase. These results make EDC crosslinking a strong candidate to protect allografts against irradiation effects. Success of EDC is likely due to the addition exogenous crosslinks prior to irradiation functioning to compensate for loss of crosslinks by free radicals. Though not completely successful, this translated to higher mechanical properties, as well as collagenase and selective trypsin resistance.

Previous studies have shown that EDC crosslinking strengthens collagen[33], however EDC crosslinking of tendon did not increase strength of control unsterilized tendon. The difference in our situation is the complexity of native tissue structure. The lack of difference may be the result of a limited number of crosslinking sites in tissue compared to pure collagen. Another explanation could be that EDC does not adequately penetrate the tissue and the majority of crosslinking occurs on the surface. This would result in high

collagenase resistance because initial degradation would have to begin on the surface. Surface crosslinking may also partially deter effects of irradiation, and only partially contribute to strength.

5.2.2 Glucose

Glucose crosslinking of rabbit tendons has been studied in the past and is associated with increased mechanical properties[56,57]. Additionally, the crosslinking reaction has been shown to be expedited by free radicals[60,61]. Our glucose crosslinking data concur with both statements. Evidence of glucose crosslinking included increased mechanical properties, specifically strength, modulus, and toughness (Fig. 4-3, 4-5, 4-7) compared to untreated. Secondly, the occurrence of crosslinking coincided with the presence of irradiation. Glucose treated tendons also displayed increases in collagenase resistance with irradiation. Increases in strength and modulus also occurred for irradiation conditions. Unirradiated glucose soaked tendons do not show any increases in mechanical properties to the control unirradiated tendons, implying a lack of crosslinking. This was to be expected because the time for natural glycation to proceed to completion is on the order of weeks to months[57]. Tendons in this study were only soaked for 36 hours prior to irradiation. Therefore the data suggests the ability of irradiation-derived free radicals to drive glucose crosslinking in tendon. Among Phase II groups, glucose crosslinking was the most resilient to irradiation in terms of mechanical properties. At 25 kGy, this

group was able to restore strength, modulus, strain, and toughness to levels of native tendon.

At 50 kGy strength and toughness seemed to be reduced, which is most likely the result of overwhelming effects of irradiation. Another possibility could be rate of crosslink breakdown by free radicals is higher than the rate of the glycation reaction. However, there is not enough known about kinetics of crosslinking reactions versus reactions free radical breakdown. Free radical formation may also be propagated by glucose in the presence of a high density of irradiation derived free radicals. Glucose has been identified as a free radical scavenger or generator depending on the circumstance[60]. The ability to perform both is common to natural multifunctional compounds, similarly for riboflavin and ascorbate as shown later.

5.2.3 Mannitol

Mannitol soaking had the least effect in the presence of irradiation, in most cases there was no difference compared to untreated. Working against mannitol is that fact that it can also participate in formation of more free radicals. Reactions between hydroxyl radicals and organic alcohols like mannitol can form superoxide anions[25]. Hydroxyl radicals generated from irradiation react with mannitol to form a mannitol radicals, which then can react with natural oxygen to form hydroxylalkyl peroxyl radicals. This radical can degenerated into an aldehyde and superoxide anion[25]. Another study reported the possibility of hydroxyl radical abstraction of hydrogen from mannitol at the C-1 or C-6 position

forming the mannitol radical[63] (Fig. 5-1). Therefore scavenging ability maybe offset by formation of radicals through this mechanism. Although solitary use of mannitol at this concentration was not successful at restoring mechanical properties, success has reported in other studies. Recall that mannitol has been successful in the past as part of a scavenging cocktail to protect tendons after gamma irradiation[10]. It is unknown whether the higher concentration used with mannitol contributed to a heightened probability of producing organic radicals. Perhaps at a lower concentration, as seen with ascorbate, mannitol effects could be more beneficial.

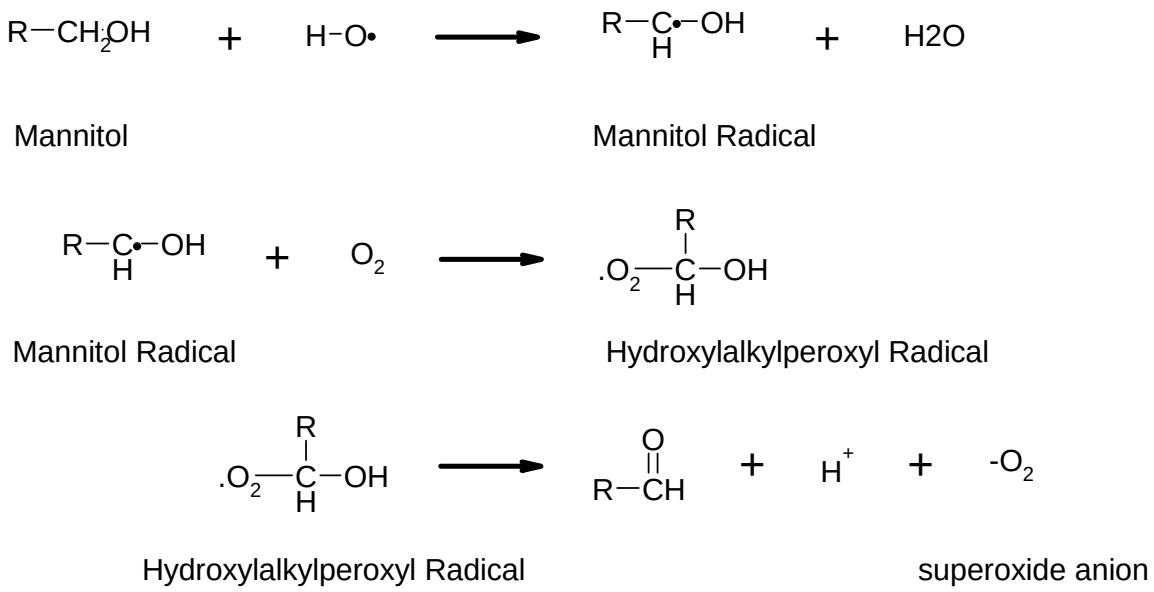


Figure 5-1 Mannitol free radical generator.

This is the pathway displaying formation of mannitol radicals by hydroxyl ions leading to the formation more free radicals (superoxide anion).

5.2.4 Ascorbate

Ascorbate has shown to provide benefit at the lower dose, which can be attributed to its potent scavenging ability. Similarly as in other treatment groups, declined benefit beyond 25 kGy may be the result of overwhelming population of free radicals at higher doses. It is possible that this could be the result of free radical propagation by ascorbate when more free radicals are present at high concentrations. Ascorbate is also among versatile compounds that can undergo both radical quenching and formation. In vivo studies have shown that vitamin C can form free radicals through the fenton reaction, which in the presence of free radicals and metallic copper can propagate more free radicals[23]. Although these are in vitro studies, a similar reaction can occur in the presence of hydrogen peroxide (H_2O_2)[23] (Fig. 5-2), which is a potential product after irradiation of water[11]. Furthermore, scavenging ability may be concentration dependent. Antioxidant studies involving ascorbate over a concentration range has shown that at higher concentrations scavenging was less efficient (Appendix Fig. A-1). After identifying this characteristic, concentration dependence was investigated at 50mM, 100mM, and 500mM. It was found that scavenging ability increased with concentration up to 100mM. At 500 mM, effects of irradiation on mechanical properties were negative and lower than untreated tendons. It is unknown whether a higher concentration of ascorbate would lead to higher probability of free radical formation through the fenton reaction.

$$2 \cdot HO_2 \longrightarrow H_2O_2 + O_2$$

Figure 5-2 Formation of hydrogen peroxide: Ascorbate free radical generator

Hydrogen peroxide can be generated from hydroxyl free radicals generated from irradiation of water. Ascorbate can undergo a reaction similar to the fenton reaction with hydrogen peroxide to form more free radicals.

5.2.5 Riboflavin

Our results suggest there is definite benefit to riboflavin treatment during ionizing irradiation. The mechanism of protection is unknown, although suggestions point toward either free radical scavenging[49], potential crosslinking[68], or a combination of both. Confirmation of either mechanism is not possible after analysis of our results. Glucose crosslinking of collagen films has been shown to be driven by UV or gamma irradiation derived free radicals[60,61]. Interestingly, it is also known that riboflavin can undergo crosslinking in collagen as discussed in the materials section[68]. Porcine cornea was crosslinked in riboflavin solution after exposure to UV irradiation[42,70]. Whether the crosslinking processes are similar or if gamma irradiation of riboflavin can cause the same effects has not been determined. However we still believe it could be a plausible cause for protection of properties. Riboflavin did show significant though selective improvement in collagenase resistance, which was seen with crosslinkers but not with other free radical scavengers. Like other free radical scavengers riboflavin displayed greater effects at the lower dose. Mannitol and ascorbate are both considered potent free radical scavengers, but riboflavin is usually not mentioned with this regard. However mechanical properties of

riboflavin are similar to ascorbate. This leaves open the possibility of supplementary effects such as riboflavin crosslinking. Riboflavin was also unsuccessful at fully restoring mechanical and degradation properties at high dose. There are several reasons that could be behind this observation. As mentioned in the materials section, riboflavin has the ability to both take up and discard electrons. Riboflavin demonstrates this in vivo as an electron transfer agent during aerobic metabolism[66]. Therefore releasing of electrons could contribute to the reformation of radical species.

Photocrosslinking ability has also been applied to collagen scaffolds for tissue engineering[71]. Crosslinking of collagen gels using photosensitizers like riboflavin has been recently achieved using rose bengal[55]. If riboflavin crosslinking is to be used as a protective treatment, it would be perhaps more successful as a pre-irradiation procedure. Until similar ability to perform riboflavin crosslinking is proven with ionizing irradiation, it would be safer to pre-crosslink with UV light as displayed in previous studies [42,68,70,84]. This would allow unimpeded crosslinking before irradiation and avoid any potential conflicting effects of free radicals, which could occur with concurrent crosslinking and irradiation.

5.3 Phase III Dual Treatments

Combination crosslinking and free radical scavenging was attempted to determine if both methods would result in better protection than individually. It was found that by bolstering crosslink density prior to and scavenging radicals

during irradiation, strength and modulus could be nearly restored to native tendon. Again strain remained relatively unchanged. Toughness however, was decreased both pre-irradiation and post-irradiation. This is likely the result of EDC crosslinked of tissues, resulting in increases for both strength and modulus, but also possible brittleness corresponding to lower toughness. EDC crosslinking followed ascorbate scavenging have successfully stabilized tendons at 50 kGy for both types of irradiation. Strength and modulus values were comparable to native tendon (Fig. 4-10). This is among the main goals sought in this research, and previously was unattainable with EDC crosslinking or scavenging alone. It seems that majority of the strength is likely due to the EDC crosslinking, with scavengers providing supplemental protection. There are several observations that suggest this. Free radical scavengers were less effective at 50 kGy than crosslinking with EDC. Comparison of EDC/MA and mannitol only treatments is even more definitive. Mannitol treatment alone had almost no effect on mechanical properties after irradiation. However when EDC is combined with mannitol treatment, there was an improvement in mechanical properties.

EDC and riboflavin did not quite show improvement to the levels of the other two scavengers for gamma irradiation. One possibility stems from potential for riboflavin induced crosslinking. Available sites for crosslinking are exhausted by initial EDC crosslinking, leaving fewer for riboflavin crosslinking. Furthermore, riboflavin scavenging ability is not as efficient as the others. There is no difference in mechanical properties between EDC/RB and EDC alone. Although it

is not known whether riboflavin does indeed form radical induced crosslinks or if active sites overlap with EDC crosslinking sites.

5.4 Limitations

There are several limitations to our study that should be noted. Mechanical testing was based on simulation of immediate failure injuries, whereas the majority of those receiving allograft replacements will be subjected to more cyclic loading[13]. Our results also cannot predict post-implantation outcomes of allografts. Only after in vivo studies are performed will one be able to make certain conclusions about post-implantation behavior. Additionally, there were no validation experiments conducted to verify gamma or ebeam ability to neutralize pathogens. Dose selection was based on past successful pathogen inactivation experiments in literature.

Collagenase and trypsin resistance testing is a useful predictor of performance after implantation, and ideally should also mirror mechanical data but was not the case. Collagenase resistance did not display significant differences in dose dependence as observed in mechanical testing. Degradation by bacterial collagenase is not limited to crosslinks, but severs the bond between ‘X’ and glycine amino acids in the repeating sequence of –X-Gly-Pro-Y- in collagen[78]. Another possible reason for a lack of sensitivity may be because the concentration used was too high. An activity of 400u/ml was used, which is about ten times the amount compared to literature studies. A higher activity was chosen to ensure the assay reaches completion in a timely manner, due to the large

amount of testing associated with this study. With the higher activity, observation of sample was done more frequently because smaller differences between dissolution times would be more significant. However, time points may not have been frequent enough to catch possible dose effects and the slight differences recorded had been deemed insignificant statistically. Although a higher concentration was used to be time efficient, it may be more beneficial to use a lower concentration over a longer study to observe changes in dissolution time. However, the small differences observed did reflect the same conclusions as mechanical tests. Trypsin determination of crosslinking could be confounded because tendons had to be initially denatured in boiling water. For example, the nature of possible riboflavin crosslinking and susceptibility to heat denaturation is unknown. Furthermore, it may be possible that trends could have been lost in trypsin testing as a result of unregulated boiling temperature. Emphasis was placed on controlling boiling time, however higher temperature could neutralize subtle trends between groups. Trypsin experiments will likely have to be redone with tight restrictions on temperature to collect more accurate data.

Differences in data may be also due to the nature of mechanical testing versus enzyme degradation and how the irradiation effects determine the results of each test. For example the extreme decrease in strength of freeze dried irradiated tendons was not as outstanding in collagenase resistance. Ionizing irradiation causes very small defects in tendon as a result of chain scission and crosslink breakdown. For mechanical tests, tendons are pulled to failure, and for enzyme tests tendons are soaked in enzyme solution. For mechanical testing,

small defects could be catastrophic enough to cause premature tearing and failure. Conversely, enzyme degradation would likely proceed at the same rate regardless of small defects. Originally collagenase and trypsin resistance testing was developed in this lab for studying biomaterials, often consisting of purely collagen. When used on native tissues several influencing factors need to be considered. These factors also may play a larger role in resistance testing than mechanical testing. Complex geometry of the tissue environment may impede enzyme penetration. Additionally, there are other non collagen components in tendon structure such as proteoglycans and glycosaminoglycans surrounding collagen fibrils as well as the connective tissue composing the tendon sheath. It is unknown if any of these components influence the rate of enzymatic digestion[85].

Despite these limitations, this study introduces novel data to tissue sterilization literature including potential of EDC and glucose crosslinking, direct comparison of crosslinking and free radical scavenging, as well as use of ebeam irradiation for these conditions.

5.5 Crosslinking vs scavenging

The two methods of modifying tendons each have their advantages and disadvantages. The advantage of using free radical scavengers is that there is a limitation on changes to structural chemistry. This avoids potential alteration of functionality and toxicity. Toxicity of scavengers was prevented by selecting from a pool of known natural and nontoxic scavengers. An associated disadvantage is

the possibility that radioprotection may encompass bacteria within the tissue as well[12], thus requiring higher doses. It has been shown that free radical scavengers can also be effective if presented immediately after irradiation[44]. This may be important because there is little known about the effect of irradiation on free radical scavengers themselves. As a post-sterilization treatment, any potential damage to scavengers would be avoided. Scavenging also cannot defend against chain scission, whereas added crosslinks may be able to bridge broken chains. Crosslinking does involve chemical modification and inclusion of spacer molecules has become an issue. The prime example has been glutaraldehyde crosslinking, which has resulted in toxicity after hydrolysis[52]. EDC crosslinking is a zero order crosslinker meaning no foreign subunits exist in crosslinks[52]. Wissink et al. has reported no issues with human umbilical vein endothelial cell attachment, morphology, or function to EDC/NHS crosslinked collagen scaffolds[86].

It is most important allografts be cleared of all possible disease transmission, and additionally preservation of mechanical and biological functions. Among Phase II groups, glucose and EDC treatment have the most potential for immediate use towards terminal sterilization. EDC crosslinked tendons sterilized at 50 kGy had notably high tensile strength and toughness, and highest resistance to collagenase. At 25 kGy, glucose treated tendons possessed comparable strength, modulus, toughness, and strain to native tendons. Comparing between crosslinkers in light of minimizing the amount of processing, as mentioned to reduce potential biological effects, shows glucose

soaking is much less extensive than EDC crosslinking procedures. Although investigation of combination of scavenging and crosslinking suggests that this will likely the best candidate for allograft protection, pending further research.

5.6 Possibilities of lowering bioburden

These results are valuable even if treated conditions could not fully protect against high doses. Calculation of terminal dose is primarily based on bioburden[43,87], which is a count of the initial pathogen density, also pathogen resistivity[11], and processing conditions to lesser extents. It is possible that bioburden is initially minimized following tissue bank safety protocols: donor history investigation, screening assays, and aseptic handling. It may also be possible to lower bioburden with decellularization[88]. The purpose for removal of cells from allografts is to reduce severity of immune response upon implantation[89]. Removal of cells and cellular components is also part of a procedure for a newly patented disinfectant process known as Biocleanse®[90]. Blood, marrow, lipids, and cells are identified as major sources for viral transmission[90]. Biocleanse® is a multiple step process in which tissues are subjected to several chemicals and detergents aimed at removing bacteria and cellular components[90]. Furthermore they have shown that mechanical properties of tissues are not compromised[91]. Although due to the novelty of this process, it is likely more research would be required to ensure sterilization effectiveness is equal to that of ionizing irradiation.

The possibility of reducing bioburden levels through decellularization methods combined with tissue bank screening protocols would reduce the terminal sterilization dose. If it is possible that the terminal dose can be lowered to 25 kGy, the different techniques assessed in this study would provide ideal contributions to stabilizing mechanical properties and resistance to degradation in soft tissue allografts. At 25 kGy, glucose treatment has already shown to have similar strength to native tendon. Additionally free radical scavengers, ascorbate and riboflavin, were also more effective at 25 kGy. This also may be a more suitable option for those who are more cautious about implantation of chemically crosslinked allografts and favor protection using natural components.

6 Summary and Future Directions

This work presented investigation into methods to stabilize allografts in the presence of ionizing irradiation at potential terminal sterilization doses. Several treatment methods and sterilization conditions were examined, and a short summary of key points may aid in organization of conclusions. Being a preliminary study and part of continuing research, future studies are also outlined from these conclusions and stated limitations.

1) Deep freezing and freeze drying have been the most common methods of allograft storage. If irradiation is part of sterilization protocol, it is highly recommended that freeze dried processing be avoided due to the extreme

mechanical weakening. Although the cause of this reduction is still unknown, our data is in agreement with several other similar studies[80,81].

2) Glucose crosslinking was able to protect tendons irradiated at 25 kGy. Mechanically, strength, modulus, strain, and toughness are comparable to native tendon and significant increases in collagenase resistance were recorded. Furthermore, our data also suggest that glycation is expedited by free radical presence in agreement with previous studies from our lab[59,61]. EDC crosslinking also displayed high strength and collagenase resistance at 50 kGy, relative to untreated and other treated groups. Terminal sterilization doses may be reduced by decellularization techniques and combined with these treatments, complete stabilization could be attainable.

3) Our results suggest the possibility of free radical derived riboflavin crosslinking in tendon allografts with exposure to ionizing irradiation. Although, explicit tests were not conducted to verify crosslink formation nor is the mechanism of crosslinking clear. Nevertheless, photocrosslinking demands more attention as another possible method of irradiation stabilization as well as other applications.

4) The best mechanical data and collagenase resistance was observed with combined EDC crosslinking and free radical scavenging. This dual treatment was able to maintain strength and modulus in tendons exposed to 50 kGy of

irradiation. Pending further research, dual treatment may also represent a method for complete stabilization at terminal doses.

Further investigation in several areas must be conducted in the next phases of research to progress this project forward. First studies regarding radioprotection by combined crosslinking and scavenging should be expanded. Successful in vitro studies should then be continued in vivo in small animals and later large animal models. Pathogen inactivation should also be verified to ensure free radical scavenging does not extend radioprotective effects to bacteria. Possible reduction of bioburden with decellularization and combination with radioprotection techniques followed by high dose sterilization should also be investigated. It is our desire that these studies will progress the application of these protective methods enabling the use of ionizing irradiation terminal sterilization purposes, which will in turn provide complete confidence in tissue allograft distribution.

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Appendix

Raw Data Phase I Mechanical testing

A) Frozen tendons exposed to 15 kGy, 25 kGy, 50 kGy Gamma and ebeam irradiation. The 15 kGy condition was not used beyond baseline studies.

Frozen

Dose (kGy)	Breakload (N)	Stiffness (N/mm)	MaxDef (mm)	Energy (Nmm)	UTS (MPa)	Modulus (MPa)	MaxStrain ----	Tough (MPa)
0								
Avg	219.76	55.45	4.33	308.85	51.07	333.51	0.17	4.74
Stdev	67.91	13.24	0.89	136.90	7.51	61.79	0.04	2.01
15 Gamma								
Avg	144.18	40.66	4.38	182.37	41.35	259.00	0.20	4.16
Stdev	40.26	15.40	0.65	41.44	10.04	70.59	0.05	1.93
15 Ebeam								
Avg	154.15	44.79	4.33	186.11	37.03	229.03	0.21	3.58
Stdev	23.53	14.50	1.03	51.06	5.99	79.68	0.06	1.62
25 Gamma								
Avg	137.17	35.07	4.32	201.08	31.04	181.31	0.18	3.25
Stdev	44.87	14.72	0.88	76.40	6.52	31.47	0.03	1.07
25 Ebeam								
Avg	112.61	27.49	4.53	165.37	34.00	208.12	0.22	3.96
Stdev	48.01	14.56	1.26	80.88	3.11	113.24	0.10	1.83
50 Gamma								
Avg	107.92	35.17	6.28	113.46	25.76	223.15	0.26	1.74
Stdev	66.63	14.14	2.17	85.33	2.35	49.15	0.09	0.32
50 Ebeam								
Avg	128.92	35.12	4.74	203.53	20.62	171.65	0.14	1.54
Stdev	69.49	38.07	1.74	138.05	3.73	66.04	0.06	0.51

Raw Data Phase I Mechanical testing

B) Freeze dried tendons exposed to 15 kGy, 25 kGy, 50 kGy Gamma and ebeam irradiation.

Freeze Dried

Dose (kGy)	Breakload (N)	Stiffness (N/mm)	MaxDef (mm)	Energy (Nmm)	UTS (MPa)	Modulus (MPa)	MaxStrain ----	Tough (MPa)
0								
Avg	180.86	38.59	5.40	294.59	44.06	329.02	0.16	3.65
Stdev	64.10	17.14	1.28	130.46	16.15	120.43	0.05	2.44
15 Gamma								
Avg	134.54	34.89	4.84	178.20	25.81	210.29	0.15	1.76
Stdev	44.47	12.35	1.01	68.81	6.05	49.28	0.03	0.51
15 Ebeam								
Avg	83.90	44.07	3.01	115.02	14.50	234.68	0.09	0.63
Stdev	17.49	10.92	0.36	16.53	3.62	61.39	0.02	0.18
25 Gamma								
Avg	83.90	44.07	3.01	115.02	14.50	234.68	0.09	0.63
Stdev	17.49	10.92	0.36	16.53	3.62	61.39	0.02	0.18
25 Ebeam								
Avg	74.21	20.90	4.16	99.81	19.98	159.86	0.16	1.59
Stdev	27.33	9.28	2.02	63.41	5.07	93.03	0.06	0.90
50 Gamma								
Avg	36.13	26.53	2.14	37.93	7.97	185.47	0.07	0.29
Stdev	13.55	10.45	0.43	20.83	2.61	38.64	0.02	0.22
50 Ebeam								
Avg	20.67	12.97	1.37	8.72	4.73	67.65	0.06	0.15
Stdev	7.92	3.80	0.45	3.91	2.26	26.72	0.02	0.10

Raw Data Phase 2 Mechanical testing

C) EDC and glucose crosslinked tendons exposed to 25 kGy and 50 kGy gamma and ebeam irradiation.

EDC

Dose (kGy)	Breakload (N)	Stiffness (N/mm)	MaxDef (mm)	Energy (Nmm)	UTS (MPa)	Modulus (MPa)	MaxStrain ----	Tough (MPa)
0								
Avg	246.42	75.08	4.67	537.77	45.47	369.31	0.18	3.83
Stdev	77.85	17.52	1.04	265.32	8.77	117.05	0.06	1.86
25 Gamma								
Avg	213.47	65.34	4.92	514.05	33.77	278.12	0.18	3.09
Stdev	77.71	22.50	1.39	264.18	8.97	67.95	0.06	1.42
25 Ebeam								
Avg	175.50	47.88	5.05	515.89	39.82	268.14	0.22	4.65
Stdev	119.08	26.08	0.74	517.72	4.10	81.08	0.05	1.42
50 Gamma								
Avg	165.54	47.23	4.14	412.47	39.70	336.05	0.15	2.93
Stdev	133.70	20.85	1.59	496.92	3.33	46.17	0.02	0.66
50 Ebeam								
Avg	152.78	47.67	4.41	353.79	30.75	245.49	0.18	2.68
Stdev	80.61	25.54	0.64	254.24	7.05	82.88	0.03	0.47

Glucose

Dose (kGy)	Breakload (N)	Stiffness (N/mm)	MaxDef (mm)	Energy (Nmm)	UTS (MPa)	Modulus (MPa)	MaxStrain ----	Tough (MPa)
0								
Avg	139.21	38.89	3.89	157.16	41.65	247.33	0.19	3.87
Stdev	88.93	24.60	0.58	101.96	5.41	72.40	0.03	1.11
25 Gamma								
Avg	243.06	68.71	4.99	580.11	51.22	413.96	0.18	4.42
Stdev	89.80	28.35	0.32	208.17	12.33	117.16	0.03	1.33
25 Ebeam								
Avg	142.77	52.43	3.77	259.83	49.17	374.79	0.19	4.45
Stdev	25.14	10.63	0.52	58.17	8.86	57.58	0.02	1.17
50 Gamma								
Avg	189.82	63.68	4.05	362.04	36.60	346.21	0.15	2.52
Stdev	90.06	27.18	0.63	196.08	6.77	82.01	0.02	0.74
50 Ebeam								
Avg	144.62	56.64	4.10	257.67	25.82	330.52	0.12	1.32
Stdev	23.47	27.07	1.58	99.11	6.86	140.77	0.02	0.30

Raw Data Phase 2 Mechanical testing

D) Tendons treated with free radical scavengers then exposed to 25 kGy and 50 kGy gamma and ebeam irradiation.

Mannitol

Dose (kGy)	Breakload (N)	Stiffness (N/mm)	MaxDef (mm)	Energy (Nmm)	UTS (MPa)	Modulus (MPa)	MaxStrain ----	Tough (MPa)
0								
Avg	176.43	45.45	4.60	243.75	34.91	244.04	0.18	3.21
Stdev	70.44	18.24	0.95	127.36	9.57	80.59	0.08	2.13
25 Gamma								
Avg	290.21	51.78	5.28	504.52	48.98	236.65	0.23	6.35
Stdev	112.02	16.41	0.60	209.04	14.82	109.54	0.10	4.14
25 Ebeam								
Avg	125.22	38.88	4.45	180.79	28.80	277.91	0.14	2.12
Stdev	33.59	16.57	0.99	80.49	3.12	51.27	0.04	0.67
50 Gamma								
Avg	175.73	41.41	5.29	304.48	25.79	126.12	0.26	3.54
Stdev	67.63	15.02	1.55	213.07	2.95	25.50	0.07	1.26
50 Ebeam								
Avg	118.86	32.40	3.76	128.80	19.41	153.52	0.13	1.25
Stdev	36.83	9.72	1.12	56.69	6.07	49.32	0.03	0.77

Ascorbate

Dose (kGy)	Breakload (N)	Stiffness (N/mm)	MaxDef (mm)	Energy (Nmm)	UTS (MPa)	Modulus (MPa)	MaxStrain ----	Tough (MPa)
0								
Avg	297.36	70.21	5.77	771.47	66.22	469.97	0.18	5.66
Stdev	74.31	19.80	1.55	428.79	6.43	39.15	0.02	1.11
25 Gamma								
Avg	215.09	72.31	4.22	468.55	41.17	363.19	0.17	3.24
Stdev	93.40	15.55	1.08	327.31	7.03	130.81	0.04	0.75
25 Ebeam								
Avg	139.80	53.56	3.68	256.46	54.46	424.03	0.20	5.35
Stdev	40.10	9.45	0.53	106.40	8.42	72.65	0.04	2.02
50 Gamma								
Avg	158.78	56.93	3.80	302.17	31.16	278.97	0.16	2.40
Stdev	63.33	14.55	0.84	215.96	8.91	76.57	0.05	1.49
50 Ebeam								
Avg	116.80	63.96	2.76	141.56	25.42	266.86	0.14	1.60
Stdev	15.98	17.42	0.29	35.72	5.66	126.00	0.03	0.49

Riboflavin

Dose (kGy)	Breakload (N)	Stiffness (N/mm)	MaxDef (mm)	Energy (Nmm)	UTS (MPa)	Modulus (MPa)	MaxStrain ----	Tough (MPa)
0								
Avg	209.01	53.68	5.04	509.84	52.08	412.27	0.17	4.28
Stdev	46.69	4.18	0.79	183.45	10.97	85.46	0.03	1.73
25 Gamma								
Avg	208.75	59.39	4.60	447.90	37.27	243.28	0.20	3.66
Stdev	61.03	18.03	1.07	191.76	11.44	63.46	0.05	2.07
25 Ebeam								
Avg	190.29	64.10	4.37	406.51	42.63	363.46	0.18	3.63
Stdev	71.41	20.97	0.78	228.07	11.22	81.44	0.04	1.85
50 Gamma								
Avg	152.23	46.14	4.75	341.71	31.59	336.47	0.13	1.98
Stdev	80.34	24.56	0.60	178.14	7.63	90.11	0.02	0.61
50 Ebeam								
Avg	136.06	48.73	3.83	234.75	28.38	329.22	0.13	1.56
Stdev	32.06	5.25	0.78	83.57	2.52	91.36	0.03	0.17

Raw Data Phase 3 Mechanical testing

E) EDC/NHS crosslinking followed by free radical scavenger soaking. Tendons were exposed to 50 kGy gamma and ebeam irradiation.

EDC/M

Dose (kGy)	Breakload (N)	Stiffness (N/mm)	MaxDef (mm)	Energy (Nmm)	UTS (MPa)	Modulus (MPa)	MaxStrain ----	Tough (MPa)
0								
Avg	217.48	69.05	6.34	472.90	44.22	431.41	0.21	2.91
Stdev	77.58	18.96	1.04	243.25	8.62	128.03	0.04	0.90
50 Gamma								
Avg	279.67	84.58	6.01	653.09	49.26	440.50	0.20	3.73
Stdev	81.62	16.36	1.23	323.90	13.80	161.66	0.03	1.82

EDC/RB

Dose (kGy)	Breakload (N)	Stiffness (N/mm)	MaxDef (mm)	Energy (Nmm)	UTS (MPa)	Modulus (MPa)	MaxStrain ----	Tough (MPa)
0								
Avg	167.61	71.22	4.74	199.88	39.23	428.49	0.19	1.63
Stdev	99.67	26.84	0.82	224.67	9.82	102.22	0.04	0.89
50 Gamma								
Avg	153.38	57.77	5.02	311.77	39.94	366.96	0.22	2.79
Stdev	73.06	17.25	1.74	360.70	11.07	140.09	0.05	1.11

EDC/AS

Dose (kGy)	Breakload (N)	Stiffness (N/mm)	MaxDef (mm)	Energy (Nmm)	UTS (MPa)	Modulus (MPa)	MaxStrain ----	Tough (MPa)
0								
Avg	235.79	85.03	6.40	448.03	41.15	474.28	0.23	2.35
Stdev	147.33	33.10	1.48	505.73	16.83	216.54	0.04	2.04
50 Gamma								
Avg	154.62	55.16	4.98	283.23	49.42	492.45	0.18	3.09
Stdev	57.60	15.99	0.58	141.94	6.55	76.31	0.02	0.73

EDC/M

Dose (kGy)	Breakload (N)	Stiffness (N/mm)	MaxDef (mm)	Energy (Nmm)	UTS (MPa)	Modulus (MPa)	MaxStrain ----	Tough (MPa)
0								
Avg	217.48	69.05	6.34	472.90	44.22	431.41	0.21	2.91
Stdev	77.58	18.96	1.04	243.25	8.62	128.03	0.04	0.90
50 Ebeam								
Avg	182.68	53.65	3.71	202.07	39.21	235.01	0.19	3.58
Stdev	90.00	23.41	0.83	140.29	8.71	99.83	0.04	1.28

EDC/RB

Dose (kGy)	Breakload (N)	Stiffness (N/mm)	MaxDef (mm)	Energy (Nmm)	UTS (MPa)	Modulus (MPa)	MaxStrain ----	Tough (MPa)
0								
Avg	167.61	71.22	4.74	199.88	39.23	428.49	0.19	1.63
Stdev	99.67	26.84	0.82	224.67	9.82	102.22	0.04	0.89
50 Ebeam								
Avg	165.53	42.01	4.84	218.38	42.71	294.21	0.18	3.49
Stdev	42.76	11.52	0.87	67.91	10.51	40.24	0.04	1.45

EDC/AS

Dose (kGy)	Breakload (N)	Stiffness (N/mm)	MaxDef (mm)	Energy (Nmm)	UTS (MPa)	Modulus (MPa)	MaxStrain ----	Tough (MPa)
0								
Avg	235.79	85.03	6.40	448.03	41.15	474.28	0.23	2.35
Stdev	147.33	33.10	1.48	505.73	16.83	216.54	0.04	2.04
50 Ebeam								
Avg	138.88	42.20	3.35	130.01	33.99	176.40	0.19	2.90
Stdev	52.83	15.01	0.73	70.80	11.12	51.11	0.02	0.88

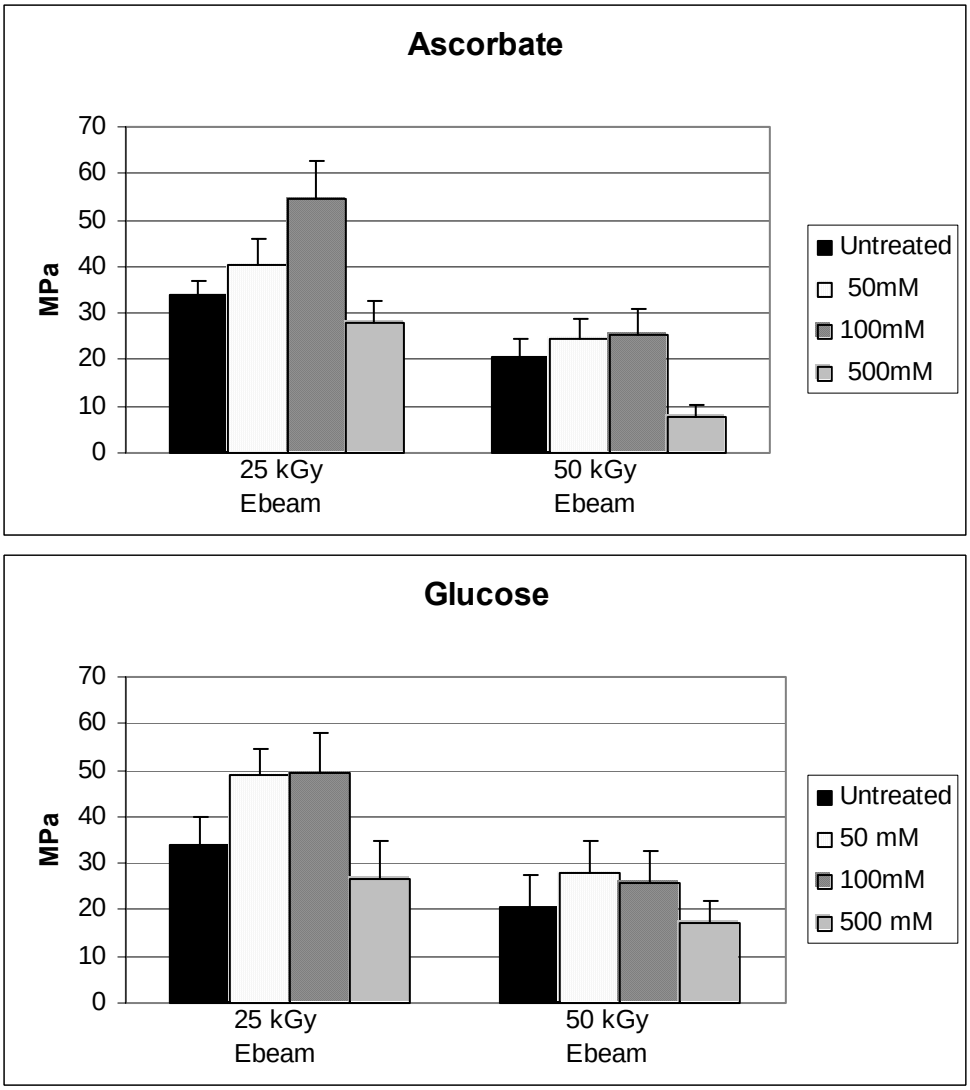


Figure A-1 Concentration dependence of Glucose and Ascorbate groups

Glucose and ascorbate treatment showed benefit up to 100mM, but at the highest concentration strength these treatments showed negative effects. These effects resulted in strength below untreated conditions.

Calculation of Mechanical Parameters using Matlab Program

F) Program 1: read smart mother board data and calculate parameters

```
%Instron - Mech Testing Template

clear all;
close all;

%Read from csv file
filename=input( 'Enter filename(.csv): ', 's');
CAL=input( 'Enter Calibration file(.csv):','s');

data=csvread(filename,5,1);
Calibration=csvread(CAL,5,1);

%Input Dimensions
W1=input('Width1(mm):');
W2=input('Width2(mm):');
Th1=input('Thickness1(mm):');
Th2=input('Thickness2(mm):');
Gauge=input('Gauge Length(mm):');
W=[W1 W2];
Th=[Th1 Th2];
CS=mean(W)*mean(Th);

%Converting volts to Load
time=data(:,1);
volts=data(:,4);
Maxvolt=Calibration(:,4);
HMaxvolt=max(Maxvolt)-mean(Maxvolt(length(Maxvolt)-10:length(Maxvolt)));
MaxLoad=1000;
Baseline=mean(volts(length(volts)-10:length(volts)));
CFactor=MaxLoad*9.8*.05/HMaxvolt;

%Calculate Load
for i=1:length(volts)
    Load(i)=(volts(i)-Baseline).*CFactor;
end

%Separate out initial Loading
for m=1:round(3/4*length(Load));
    if abs(Load(m)-Load(m+1))<1;
       Load(m)=0;
   else
       break
   end
end

%Locate First NonZero Value
for j=1:length(Load)
    if Load(j)~=0;
        Y(j)=j;
        break
    end
end

%Isolate Relevant Indices of Load
Imin=max(Y)-1;
Load=Load(Imin:length(Load));
Deformation=(100/60).*(time(Imin:length(time))-time(Imin));
```

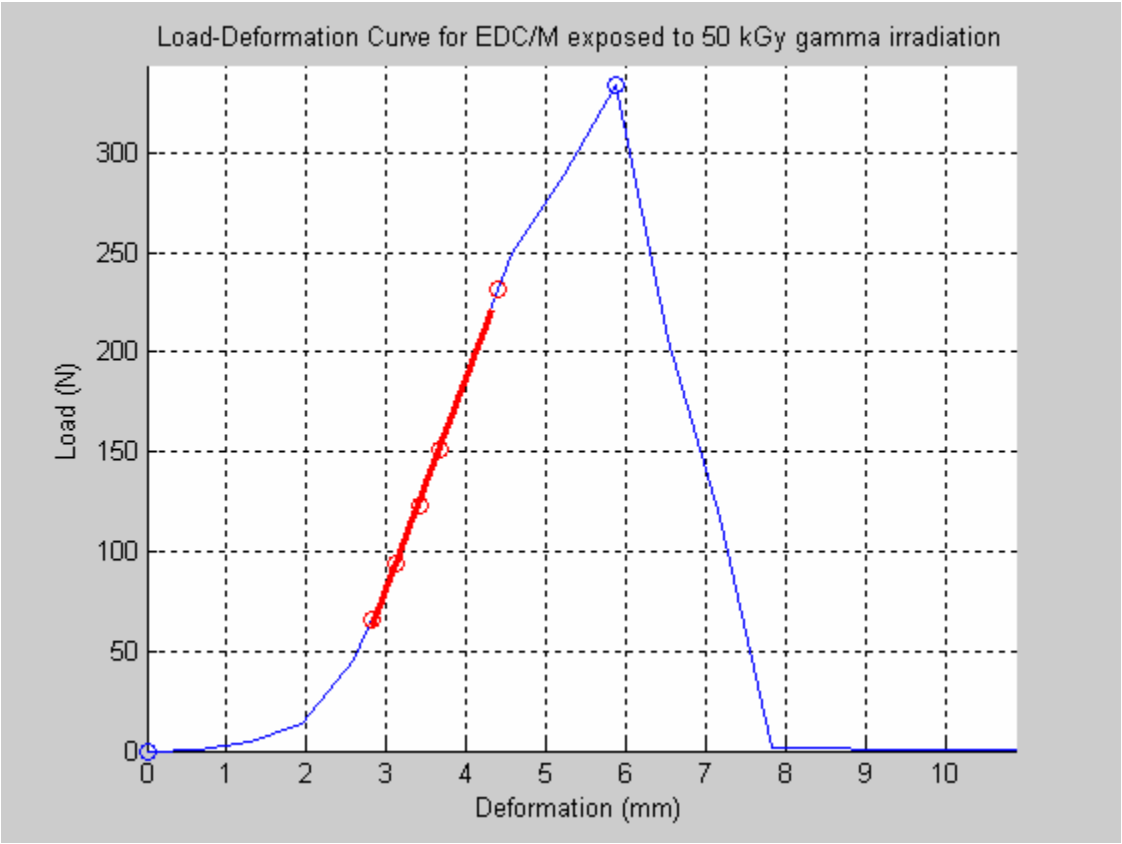
```
[breakload,Imax]=max(Load);

%Calculate Stress, Strain
Stress=Load./(CS/(1000^2)*1e6);
Strain=Deformation./Gauge;
UTS=max(Stress);

%Plotting
Plots=[zeros(1,2)];

%Load Deformation
figure
hold on
plot(Deformation(1),Load(1),'ob',Deformation(Imax),Load(Imax),'ob')
Plots(1)=plot(Deformation,Load)
title('Load Deformation')
xlabel('Deformation')
ylabel('Load (N)')
axis([0 Deformation(Imax)+5 0 breakload+10])
grid on
disp('<<Stiffness approximation>>')
disp('<<Select 5 points on curve>>')
[a,b]=ginput(5);
plot(a(1),b(1),'or',a(2),b(2),'or',a(3),b(3),'or',a(4),b(4),'or',a(5),b(5),'or')
%Least Sq Approx.
A=[a ones(1,length(a))'];
C=A'*A;
X=inv(C)*A'*b;
x=[min(a):.1:max(a)];
y=X(1)*x+X(2);
plot(x,y,'r','Linewidth',2.5)

%Stress vs Strain
figure
hold on
plot(Strain(1),Stress(1),'ob',Strain(Imax),Stress(Imax),'ob')
Plots(2)=plot(Strain,Stress)
title('Stress vs Strain')
xlabel('Strain')
ylabel('Stress (MPa)')
axis([0 Strain(Imax)+1 0 UTS+10])
grid on
disp('<<Modulus approximation>>')
disp('<<Select 5 points on curve>>')
[c,d]=ginput(5);
plot(c(1),d(1),'or',c(2),d(2),'or',c(3),d(3),'or',c(4),d(4),'or',c(5),d(5),'or')
%Least Sq Approx.
B=[c ones(1,length(c))'];
D=B'*B;
X1=inv(D)*B'*d;
x1=[min(c):.1:max(c)];
y1=X1(1)*x1+X1(2);
plot(x1,y1,'r','Linewidth',2.5)

%More Values
StrainEnergy=trapz(Deformation(1:Imax),Load(1:Imax));
Toughness=trapz(Strain(1:Imax),Stress(1:Imax));
MaxDef=Deformation(Imax);
MaxStrain=Strain(Imax);
Modulus=X1(1);
Stiffness=X(1);
```

```
%Display Values
output=[breakload Stiffness MaxDef StrainEnergy UTS Modulus MaxStrain Toughness CS];
disp(['BreakLoad = ' num2str(output(1))])
disp(['Stiffness = ' num2str(output(2))])
disp(['MaxDef = ' num2str(output(3))])
disp(['StrainEnergy = ' num2str(output(4))])
disp(['UTS = ' num2str(output(5))])
disp(['Modulus = ' num2str(output(6))])
disp(['Strain = ' num2str(output(7))])
disp(['Toughness = ' num2str(output(8))])
matfile=input('saveas(exclude ".mat") : ', 's');

%save workspace & figures
for label=1:2
   fig=num2str(label);
   figsave=strcat(matfile,fig);
   saveas(Plots(label),figsave, 'fig')
end
save(matfile);
```

Program 2: Read test files and concatenate values into single matrix

```
clear all;
close all;
disp('Loading file data')
N=input('Number of files: ');
values=zeros(N,9);
for aa=1:N
   filename=input('Enter Datafile: ','s');
   load (filename)
   values(aa,:)=output;
   clear output;
end

workspace=input('saveas: ', 's');
save(workspace);
```

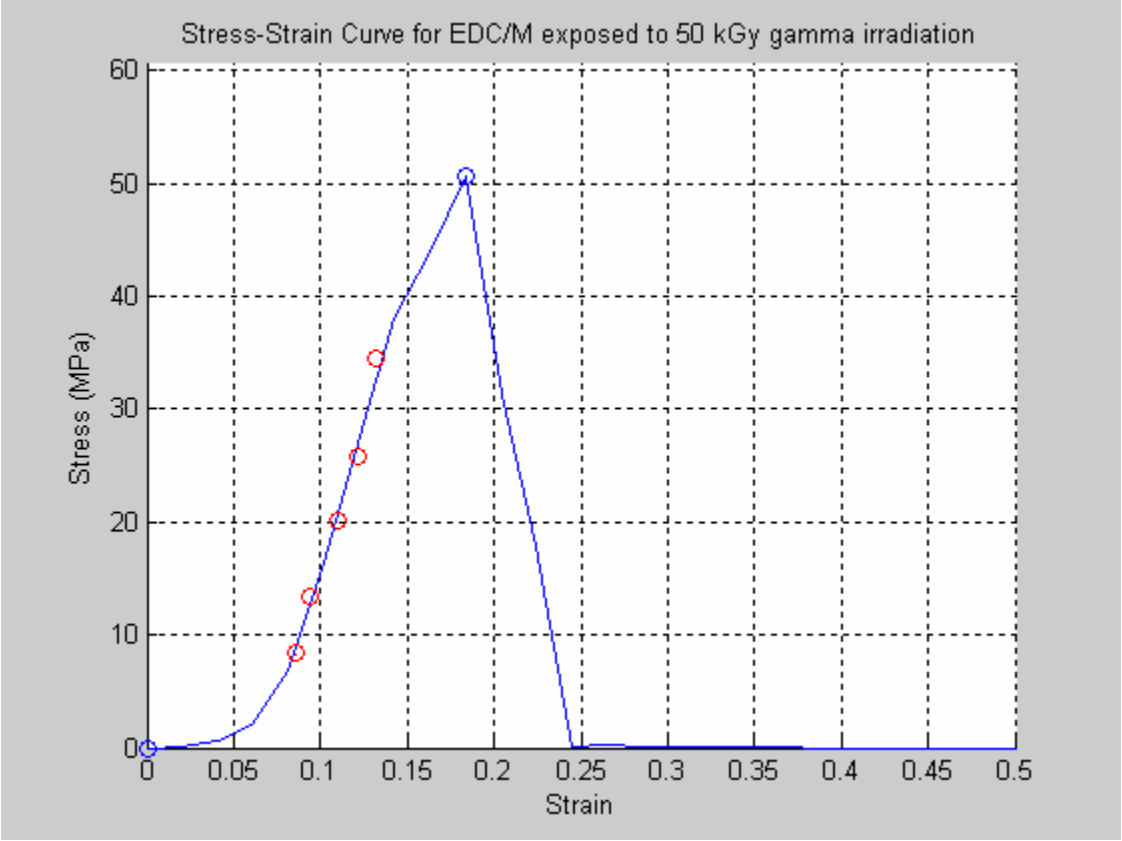
G) Sample Load-Deformation curve generated in Matlab



Calculation of Structural Properties:

- Zero value and **break load** are circled
- **Deformation** is calculated as the distance from the zero value to break load
- **Stiffness** is calculated as the slope of the linear region
- **Energy** is calculated as the area under the curve between the zero value and break load

H) Sample Stress-Strain curve generated in Matlab



Calculation of Material Properties:

- Zero value and ultimate **stress** are circled
- **Strain** is calculated as the space from the zero value to maximum stress
- **Modulus** is calculated as the slope of the linear region
- **Toughness** is calculated as the area under the curve between the zero value and maximum stress