EFFECT OF X-RADIATION ON BIOMECHANICAL PROPERTIES OF BOVINE LIGAMENTUM NUCHAE

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ABSTRACT

The extracellular matrix (ECM) is a complex structural entity surrounding and supporting cells that are found within mammalian tissue. This study presents the effect of ionizing radiation on the physical properties of elastin which is usually found within arteries, lung, skin, ligantum nuchae, vocal chords and elastic cartilage as a function of their composition and organization or architecture. X-ray from an electron linac were used to give doses of 10-50Gy to cover the range of irradiation exposure during radiotherapy. A uniaxial mechanical testing protocol was used to characterize the fibrous protein. For pericardial the major change was an increase in the elastic modulus in the toe region of the curve (≤ 20% strain), from 23±18 kPa for controls to 57±22 kPa at a dose of 10 Gy (p=0.01, α=0.05). At a larger strain (≤ 20%), the elastic modulus in the region decreased from 1.92±0.70 MPa for control pericardium tissue to 1.31±0.56 MPa (p=0.01, α=0.05) for 10 Gy X-irradiated sample. For elastin, the stress-strain relationship was linear up to 30% strain, but the elastic modulus decreased significantly with irradiation (controls 626±65 kPa, irradiated 474±121 kPa (p=0.02, α=0.05). The results suggest that for elastin chain scissions are the primary effect of irradiation. The Raman microspectrometry was employed to characterize these changes on ECM conformation.

ABSTRAK

Matriks luar sel (ECM) merupakan struktur entiti kompleks yang mengelilingi dan menyokong sel-sel yang dijumpai dalam tisu mamalia. Kajian ini menunjukkan kesan sinaran mengion terhadap sifat fizikal elastin yang biasanya ditemui dalam arteri, paru-paru, kulit, ligantum nuchae, pita suara dan rawan elastik sebagai fungsi komposisi dan organisasi atau pembinaannya. Sinar-X dari elektron linac telah digunakan untuk memberi dos 10-50Gy yang merangkumi julat pendedahan penyinaran semasa radioterapi. Protokol ujian mekanikal ekapaksi telah dijalankankan untuk mencirikan serat protein. Untuk perikardium, perubahan utama ialah peningkatan dalam modulus elastik di rantau kaki lengkung (≤ terikan 20%), dari 23 ± 18 kPa untuk kawalan ke 57 ± kPa 22 pada dos 10 Gy (p = 0.01, α = 0.05). Pada terikan yang lebih besar (≤ 20%), modulus elastik di rantau ini menurun dari 1,92 ± 0.70 MPa untuk tisu perikardium kawalan 1,31 ± 0,56 MPa (p = 0.01, α = 0.05) untuk sampel sinar-X 10 Gy. Bagi elastin, hubungan tegasan-terikan adalah linear sehingga dengan terikan 30%, tetapi modulus elastik menurun dengan ketara dengan penyinaran (kawalan 626 ± 65 kPa, sinaran 474 ± 121 kPa (p = 0.02, α = 0.05), Keputusan ini mencadangkan bahawa pemutusan rantaian elastin adalah kesan utama penyinaran. Raman mikrospektrometri telah digunakan untuk mencirikan perubahan bentuk ke atas ECM.

Keywords: Bovine Ligamentum Nuchae; Elastin; Stress-strain relationship; Raman Spectroscopy

INTRODUCTION

Elastin is a protein found in vertebrates. It is present as thin strands in the skin and in areolar connective tissue. Elastic fibers are found within arteries, lung, skin, ligamentum nuchae, vocal chords, and elastic cartilage (LB Sandberg & Leslie 1981). Elastin comprises approximately 90% of the elastic fiber and forms the internal core. It is interspersed with and surrounded by a sheath of unbranched microfibrils with an average diameter of 10–12nm (Ramirez 2000). The microfibrils are composed of a complex array of macromolecules. It forms quite a large proportion of the material in the wall of arteries and veins, especially near the heart. It is also a prominent component of lung tissue (L Debelle 1999). The structure of elastic matrices differs among tissues. Their function is a consequence of their composition and organization or architecture. Elastin constitutes 30–57% of the aorta, 50% of elastic ligaments, 28–32% of major vascular

vessels, 3–7% of lung, 4% of tendons, and 2–5% of the dry weight of skin (Vrhovski & Weiss 1998). The ligamentum nuchae, which for example runs along the top of the neck of horses and cattle, is almost pure elastin (Fung 1993). In the present investigation, specimens of bovine ligamentum nuchae representing almost pure elastin were obtained from a local abattoir. The ligament also contains a small amount of collagen which can be denatured (Daamen 2001). Young's Modulus provides a measure for the elasticity or stiffness of a material (i.e., the greater the Young's Modulus, the stiffer the material). The Modulus is determined from the slope of a stress/strain curve. The slope of this curve for elastin remains linear to an extension of 70% (John M. Gosline 1979). The Young's Modulus of elastic fibers is 300–600 kPa; for collagen, it is 1 x 10^6 kPa. The maximum extension of elastic fibers ranges from 100–220% (Fung 1993). Elastic fibers are able to undergo billions of cycles of extension and recoil without mechanical failure (Fred 2002).

EXPERIMENTAL METHOD

Bovine ligamentum nuchae was extracted using the method of Lansing (Lansing 1952) in which samples are refluxed in acetone and ether before boiling (at 66^{o}C or above) in 0.1M sodium hydroxide for 45 minutes to remove collagen and glycoproteins. Heating to this degree and cooling again does not change the mechanical properties of elastin. In preparation for mechanical testing, the tissue was then dissected into strips of approximately 21 – 25 mm in length and 1 – 2 mm in width and kept in normal saline at pH 7.2 in the presence of sodium azide. Elastin specimens were tested in a uniaxial tensile apparatus constructed in our laboratory. The quasi-static mechanical properties of bovine ligamentum nuchae were characterised by force-extension and stress-strain curves. The ends of the tissue strip were clamped between two sets of jaws. The upper jaws were attached to a force transducer (TRN 001; range 0.05 - 0.2 N. Kent Scientific Corp.) and the lower jaws to a movable lower arm connected to a micrometer screw driven by a stepper motor (RS Components, UK), displacement being measured by a displacement transducer (Research Instruments Ltd, UK). Force and displacement were logged using Picolog instrumentation (Pico Technology Ltd). The sample was extended to a force of 0.3 N, which is comparable to that experienced by the tissue *in vivo* (Winlove et al. 1996) and the length of the sample was recorded. Measurements of force and extension were carried out at rate 0.04 mm s^{-1} and were made after cyclically stretching and relaxing the sample until quasi–steady conditions were attained (this being referred to as pre-conditioning). The specimens were immersed in a saline-filled water bath, pH 7.2, thermostatted to 25^{o}C throughout testing. The same samples were tested before and after irradiation. A 6 MV x-ray beam (a Varian 2100C electron linear accelerator (linac) gave uniform doses of 10 -

50 Gy to the elastin, to cover the range of radiation doses typically delivered during radiotherapy courses (Dieckman et al. 2003; Tzedakis et al. 2004).

In earlier investigations by others, involving strips of ligamentum nuchae irradiated with x-rays, mechanical changes were observed (Bailey 1968). The strips were stretched intermittently by sinusoidal movement at a chosen frequency, and the dynamic stress-strain diagrams were registered using an oscilloscope. At 20 Mrads a progressive decrease in rigidity occurred, chain scissions being the predominant process. At extremely high dose the fibers became brittle and of low tensile strength.

Figure 1 indicates the highly linear stress-strain response of bovine nuchal elastic tissue when subjected to uniaxial mechanical testing up to 15% strain at the seventh conditioning cycle. The abscissa is the tensile strain defined as the change in length divided by the initial (unloaded) length of the specimen. The ordinate is the stress, defined as the load divided by the initial cross-sectional area of the specimen at zero stress. It is noted that the loading curve is almost a straight line. Loading and unloading leads to two different curves, resulting from the small amount of hysteresis (measured as the loop area) but the difference is small. For this tissue sample, Young's modulus is estimated as 626 ± 65 kPa.

At higher shear rates the chains cannot adjust as rapidly as the oscillating strain and thus are deformed sinusoidally.

RESULT AND DISCUSSION

Effect of X-radiation on Biomechanical Properties of Bovine Ligamentum Nuchae

Studies show that elastin can undergo more than 200% strain, with Young's modulus exceeding 250 kPa (Fung 1993; Mithieuxa 2004).

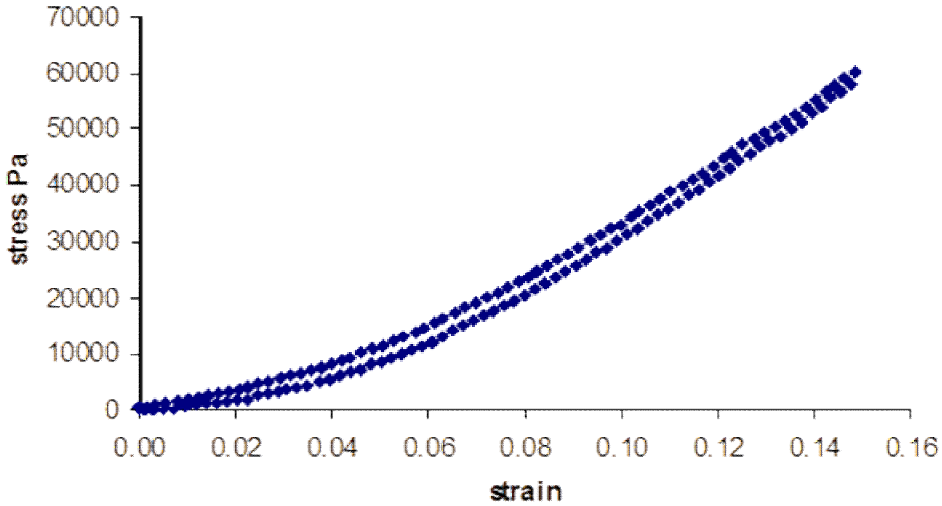


Figure 1: The linear stress-strain response of bovine ligamentum nuchae for values up to 15% strain.

The graph in figure 1 shows the linear stress-strain response of bovine ligamentum nuchae for values up to 15% strain, obtained at the seventh conditioning cycle during cyclic loading and unloading. Young's modulus is estimated to be 626 ± 65 kPa calculated from the tangent of the stress-strain curves at stretching. Each single bundle of fibre was pre-conditioned up to seven repeated cycles at 2.4mm/min constant strain rate in 0.15M isotonic saline at room temperature.

The stress-strain relationship when the tissue was subjected to uniaxial mechanical testing was linear up to 30% strain with a short toe-region, as shown in Fig.2. The elastic modulus decreased significantly with 10 Gy x-irradiation [controls 626 ± 65 kPa, irradiated 474 ± 121 kPa ($p=0.02$, $\alpha=0.05$)]. Similar decreases of elastic modulus were observed when doses were increased up to 30 Gy as indicated in figure 3.

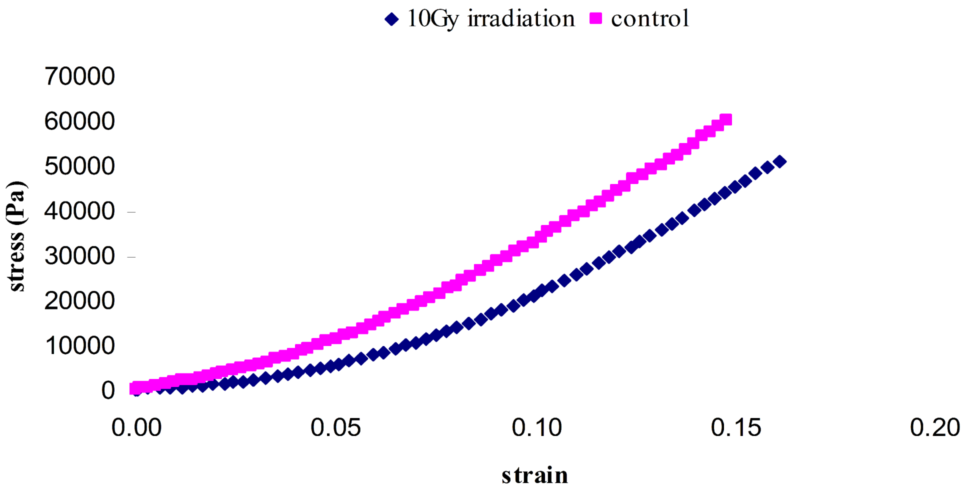


Figure 2: The stress-strain behaviour of elastin from bovine ligamentum nuchae.

The same samples were used before and after exposure to 10 Gy x-rays. The elastic modulus decreased significantly following 10 Gy x-irradiation. The data for each fibre specimen were those obtained following pre-conditioning up to seven repeated cycles at 2.4 mm/min constant strain rate in 0.15M isotonic saline at room temperature.

Further investigations were made by irradiating specimens of ligamentum nuchae over a range of x-radiation doses delivered using the same 6 MV linac source as above. Every specimen result represents that obtained for a group of three individual specimens given doses of 10 Gy, 25 Gy and 50 Gy of x-rays. Samples were then taken back to the lab to be biomechanically tested using the uniaxial mechanical testing apparatus.

The stress –strain curves were plotted using data taken from the 7th conditioning cycle. The tangents of the stress-strain curve were calculated to estimate the value of elastic or Young's modulus. The results are shown in table 1. The previously noted decrease in modulus value is again observed. However, in addition, within the range of doses delivered there is no dependence of the value of Young's modulus on the amount of dose given.

All elastin samples tested were indicating the decrease in the value of elastic modus after irradiation. Changes were significant at ($p=0.02$ at $\alpha=0.05$) confirms by ANOVA. The effect of ionizing radiation to elastin mechanics may suggest that chain-scission or degradations were predominant effect to elastin fibers as reported in the previous study (Bailey 1968). The increasing dose of radiation seems did not significantly change the decreasing value of elastic modulus but it may indicates that even at low doses of 10Gy changes of elastin mechanics can be detected (Jellinek 1962).

Table 1: Shows the value of elastic modulus estimated from the tangent of the linear stress-strain curve, the samples consisting of a bundle of fibres for each group pre-conditioned up to seven repeated cycles at 2.4 mm/min constant strain rate in 0.15M isotonic saline at room temperature.

Sample/ Radiation Dose (Gy)	Elastic Modulus (kPa)
Control	567±33
10	482±25
25	508±25
50	473±41

In contrast to the effect observed with collagen, for elastin with its much looser structure than collagen, the chains are too far apart to allow extensive cross-linking. If the elastin is irradiated in the dry state (not in the hydrated state, as in the present investigation), the probability of two reactive groups meeting is greatly increased, assuming sufficient mobility is maintained. On the other hand, in collagen, the packing may be sufficiently great that the probability of cross-linking will be maximal, and in addition the removal of water will decrease the mobility of the molecules to a sufficient extent that cross-linking is inhibited (Bailey 1968).

However, on irradiation of thermally denatured collagen, which like elastin exists in the random chain form and is similar to elastin by virtue of its natural cross-links, both wet and dry samples show a decrease in tensile strength. Although this is not an accurate comparison, it may indicate reasons for a decrease in tensile strength are more fundamental than the distance of separation of the chains. A large proportion of a polar residue is present in elastin and there is a possibility of hydrophobic centers occurring; this may affect the ability of the polar amino acids to cross-link. No specificity in amino acid degradation has been detected and the cross-links in elastin do not appear to be particularly radiation sensitive (Bailey et al. 1964).

For elastin, the effect of radiation has been observed to weaken the tissue, again presumably due to chain breakage (Bailey 1968). This process would obviously weaken tissues such as ligaments which are almost entirely elastin. In more complex structures such as blood vessels, the effects of radiation could be more complex (Jacobs 1998).

Raman spectra for ligament elastin were obtained from hydrated unirradiated tissue and tissue irradiated to a dose of 30 Gy of high energy x-rays from a linac source. For every preparation, spectra were collected from six samples, the spectra being an average for each sample, obtained over several runs taken at different locations on the specimen. Before averaging, each spectrum was checked to ensure removal of any recorded cosmic ray events. The background was subtracted from the overall spectrum, applying a cubic spline baseline through points where no vibrational modes are to be expected.

Figure 3 shows Raman spectra for bovine ligamentum nuchae in the intact state and after the tissue was exposed to an x-ray dose of 30 Gy. Analysis of the Raman spectra revealed a water peak at 3200 cm^{-1} from the interstitial water, dominated by the OH vibrations in the elastin fibers, similarly for both in control and irradiated tissues.

In the control elastic tissue, two spectral features that can be assigned to the vibrational modes of CH_2 were observed, the prominent feature being at 2943 cm^{-1}, with a shoulder at 2884 cm^{-1}. In the irradiated tissues the peak heights of CH_2 vibrations relative to water peak was smaller and CH_2 vibrational modes were observed at 2937 cm^{-1} and 2878 cm^{-1}.

The conformation-sensitive band of amide I for control ligamentum nuchae starts at 1637 cm^{-1}, achieving a maximum value of the peak at 1667 cm^{-1}. In irradiated pericardium the peak was shifted to 1659 cm^{-1}.

Significant changes observed in the amide I are mainly to be attributed to the protein-radiation interaction. The α-helix shoulder at 1600 cm^{-1} in the control tissue suffers a minimal shift to 1602 cm^{-1} in the irradiated samples (Debelle et al. 1995).

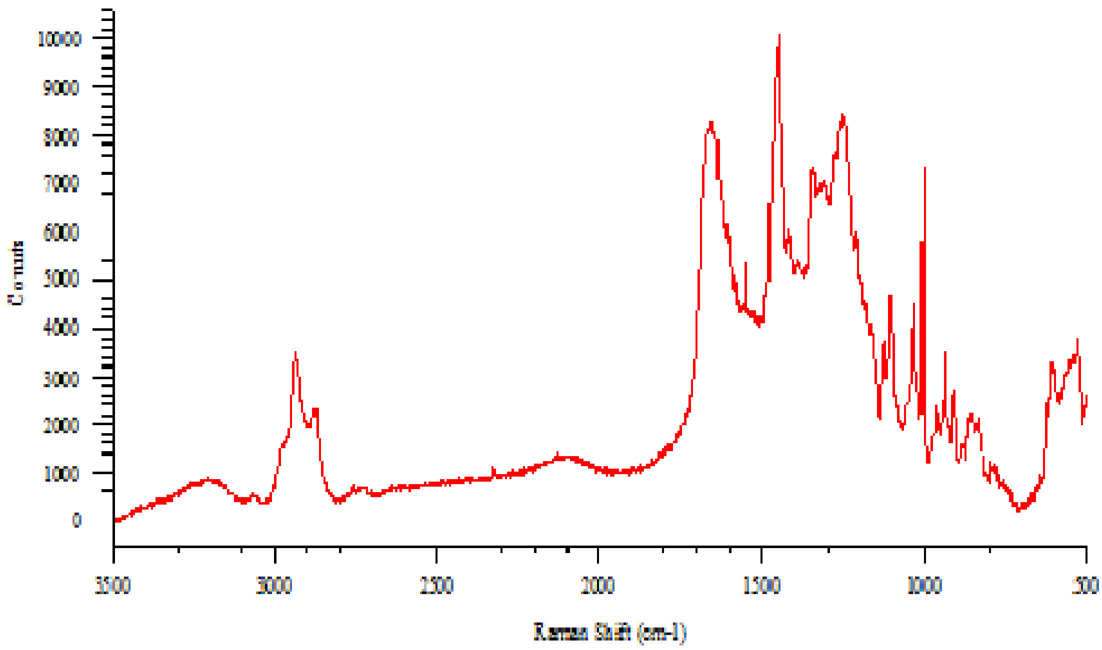
For control tissue, the CH_2 deformation band is at 1449 cm^{-1} while in irradiated tissue it is to be found at 1453 cm^{-1}. A weak peak assigned to CH_2 def was seen at 1429 cm^{-1} in control tissue while in irradiated tissue it was observed at 1417 cm^{-1}.

For the control bovine ligamentum nuchae, a substantial amount of β-structures is in evidence from the amide III band (1206 cm^{-1}). For samples irradiated to an x-ray dose of 30Gy, this peak was at a frequency of 1209cm^{-1} (Sane 2004) . Here one can further observe that structural changes in amide III region are not significant. The strong peak assigned to the Phe vibrational mode at 1006 cm^{-1} is enhanced in irradiated elastin (Debelle et al. 1995).

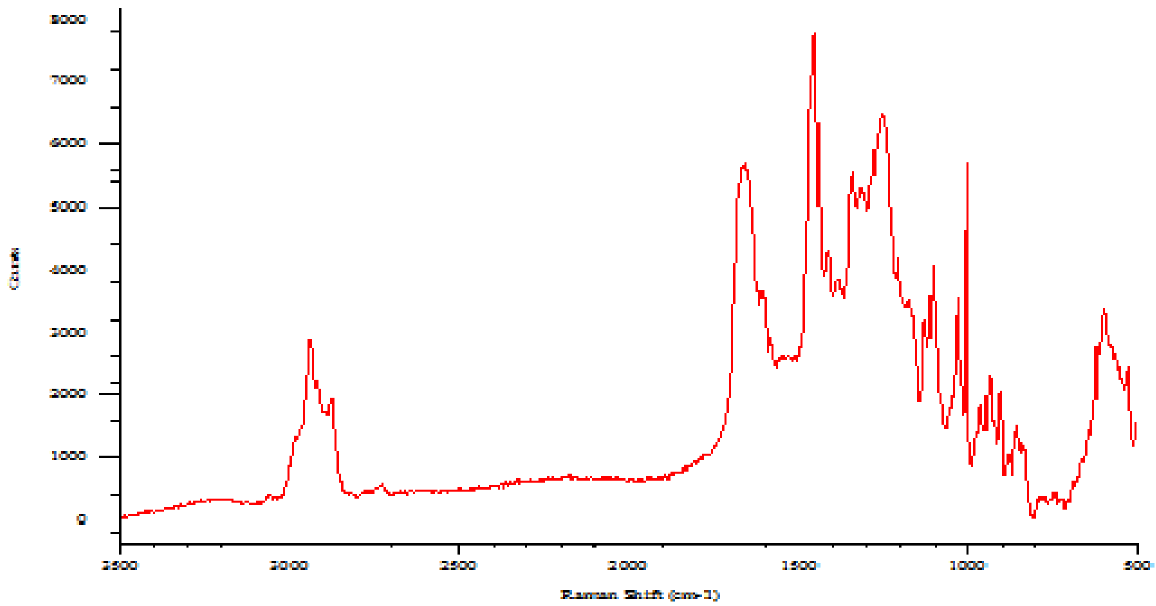
Analysis of the Raman spectrum of bovine elastin reveals that helical structures are present in the conformation of the control elastin molecule, the band at 939 cm^{-1} being typical of α- helices while for irradiated tissue the feature is observed at 937 cm^{-1}. The feature at 600 cm^{-1} in irradiated tissue are associated with Phe (Frushour 1975) and also attributed in the fingerprint region (~ 900 – 600 cm^{-1}) (Nauman 2010) is enhanced when compared to the situation in control samples. In this case, radiation induced cross linking is suspected to occur.

Raman Spectrometry Study on Bovine Ligamentum Nuchae

The C-C-C def (deformed) vibrational mode can be seen at 543cm^{-1} for control HA. In irradiated HA there is a prominent peak at 599 cm^{-1}, resulting either from a peak shift of the CCC def peak or from a skeletal vibration mode of HA. Additional peaks in the irradiated samples that can be ascribed to radiation crosslinking on hyaluronan were suspected to be found within peak 600-900 cm^{-1}.



(A) Control



(B) 30 Gy

Figure 3: Raman spectra in the range 500 – 3500 cm^{-1} for: (A) the control sample, and (B) the 30 Gy x-ray irradiated bovine ligamentum nuchae.

CONCLUSION

For elastin the effect of radiation is also known to weaken the tissue within which it is found, again presumably due to chain breakage (May-Newman 1995). This process would obviously weaken tissues such as ligaments which are almost entirely elastin. In more complex structures such as blood vessels the effects of radiation could be more complex (Bandekar 1992). In normal tissue elastin provides an initial compliance before higher loads are transferred to a collagenous network. In the present study, radiation has changed the mechanical property of elastin by the observed decrease in the Young's modulus without depending on the dose given. Damage to elastin could therefore result in a premature apparent "stiffening" of the tissue which could seriously impair its physiological performance.

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