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The effects of irradiation and hydration upon the mechanical properties of tendon

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Irradiation sterilization is in wide use among tissue banks, for both hard and soft tissue grafts. Irradiation of tendon can impair its mechanical properties. Following implantation of a tendon graft, re-vascularization and resorption processes reduce its mechanical performance. Tendon with severely impaired properties may not be suitable for use as a load-bearing graft, e.g. as anterior cruciate ligament replacement. An important factor determining the extent of the reduction of the mechanical performance is the condition of the tendon during irradiation, especially the presence of water. There has not yet been a study of the effects of both irradiation dose and hydration on tendon mechanical properties. This study measured the changes in tensile mechanical properties, including strength and stiffness, following γ irradiation doses of 15 kGy (1.5 MRad) and 25 kGy (2.5 MRad), in the frozen state and following freeze-drying. The strength of the freeze-dried irradiated tendons was lower compared to fresh tendons, whereas the strength of the frozen irradiated tendons was very similar to that of the fresh. The tangent modulus of both of the freeze-dried irradiated groups were lower than the fresh tendons, as was the 15 kGy frozen group. The modulus of the 25 kGy frozen irradiated group was similar to the fresh. The general pattern of the results indicate that the two freeze-dried tendon groups were more affected than the frozen irradiated, and of the frozen irradiated groups the 25 kGy group was least affected. The results fit well with suggested mechanisms for the action of irradiation upon collagen; that intramolecular crosslinking and scission of the tropocollagen α chains occur when water is present, and α chain scission alone occurs when water is absent. Irradiation of tendons for use as grafts may produce minimal deleterious changes if the irradiation is performed while the tendon is frozen with water present.

1. Introduction

Irradiation is in wide use among tissue banks as a method of terminal sterilization for tissue grafts. Experimental studies have shown that irradiation can impair the mechanical properties of tendon [1–3]. The doses most commonly used for sterilization lie between 10 and 30 kGy (1–3 MRad). At present irradiation is usually performed while the tissue is frozen in water, although in the past it was sometimes performed following freeze-drying of the tissues. The other common sterilization method in use, although more common in the UK than in the USA, is exposure to ethylene oxide gas. Following implantation of a graft there is a re-vascularization and collagen resorption process which reduces the mechanical performance of the graft considerably [4, 5]. It follows that the mechanical properties of tendon grafts should be as good as possible. Tendon with impaired mechanical properties may not be suitable for use as a load-bearing graft, such as an anterior cruciate ligament replacement.

The effects of irradiation upon collagen in tendon have been demonstrated by Bailey and co-workers and by others [6–9]. They have suggested that two reactions occur in collagen during irradiation, (i) a peptide bond scission along tropocollagen α chains, caused directly by ionizing particles, and (ii) crosslink formation between tropocollagens in the microfibril, caused by free radicals present in the surrounding fluid or through increased chain mobility during irradiation. Collagen irradiated without fluid present will show only the results of the first reaction, i.e. α chain scission, and collagen irradiated with fluid present will show the results of both the first and

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second reactions, i.e. α chain scission and crosslink formation. Bailey's results of increased solubility and lowered shrink temperature upon irradiation in the dry state, and decreased solubility and increased shrink temperature in the wet state fit well with the two proposed reactions [7, 8]. Importantly the maximum tensile load sustained by the tendons was also seen to negatively correlate with the changes in solubility and shrink temperature.

Many of the reports of tendon mechanical properties and the effects of irradiation in the literature are of tests done on parts of or whole patellar tendons [1, 10, 11]. Presumably these investigators used the patellar tendon for mechanical tests because it was the most common tendon used in ligament replacement operations. However, the patellar tendon is not the ideal specimen for mechanical tests; it is short, very thick and is usually taken with two blocks of bone on either end. This makes accurate measurement of the cross-sectional area very difficult. The bone blocks may be used to grip the specimen in the testing machine but precautions must be taken to ensure that the mechanical properties of the tendon are measured in isolation from the properties of the bone. Animal or human tendons can be chosen which are much better suited to mechanical testing, ideally being long and thin. Accurate measurements of the cross-sectional area can be taken using the gravimetric method of Ker [12] and the effect of the machine grips can be calculated and corrected for [13].

Nevertheless those reports of tests which have used more suitable tendons, in general, agree with those of tests upon human patellar tendons. They have found that irradiation while water is present can reduce the stiffness and strength slightly, whereas without water the strength and stiffness is reduced dramatically [3, 11, 14, 15]. There are no reports in the literature of the effects of both hydration level and irradiation dose upon tendon mechanical properties. Apart from Bailey's simple tests there are no reports in the literature on the mechanical effects of hydration together with irradiation dose. Freezing and freeze-drying by themselves have been shown not to affect the mechanical properties of tendon [2, 3, 10, 12, 14, 16].

This study reports the effects of dose and hydration level upon some of the mechanical properties of tendon. The effects of irradiation with and without water are discussed in relation to the proposed mechanisms of intermolecular crosslinking in tendon collagen put forward by Bailey and co-workers [6–8]. It is proposed that the intermolecular crosslinks identified by Bailey and co-workers [6–8] influence the strength and stiffness of tendon – a collagenous composite.

2. Materials and methods

Porcine toe extensor tendons were dissected out of pig feet obtained from a butcher one or two days *post mortem*. The tendons were typically between 100 mm and 120 mm long and between 5 and 12 mm^2 in cross-section when dissected. The tendons were then measured and weighed so as to calculate the cross-sectional area using the gravimetric method [12]. Tendons were placed with excess saline in plastic bags, sealed and frozen in a domestic freezer at $-20\,^{\circ}C$. Freezing does not radically alter the mechanical properties of tendon [3, 12, 16]. The tendons were then divided up at random into five groups and four of the groups transported on dry ice to the irradiation facility. The return journey from the irradiation facility was also on dry ice. Group 1 received no irradiation, group 2 received 15 kGy of γ radiation while still frozen, group 3 received 25 kGy γ radiation while frozen, group 4 was freeze-dried and then received 15 kGy γ radiation and group 5 was freeze-dried and then received 25 kGy γ radiation. The irradiation dose rate was 8.8 kGy/h, from a cobalt 60 γ source. The temperature and pressure during freeze-drying were maintained at approximately $-25\,^{\circ}C$ and 10 Pa. Freeze-drying took place over 5 days. The dose and hydration state during irradiation are shown in Table I.

TABLE I The mean and standard deviation of the properties measured in the tests. The second row of each group gives the statistical significance of the data compared to the fresh group's data. A significant difference (at $p < 0.05$) is marked 'yes'. The preferred statistical test was a one-way ANOVA followed by Dunnet's post-test with the fresh group as control. Where the sample size is too small or the data was not normal a Kruskall–Wallis test followed by a Dunn's post-test with the fresh group as control was used. Where small sample size prevented a test the group is marked 'no test'.

Group	UTS (MPa)	Tangent modulus E (GPa)	Fail strain (%)	Hysteresis (%)	Failure energy area (kJ/m^2)	Failure energy vol (kJ/m^3)
Fresh	49.83 (8.69 $n = 4$)	0.980 (0.12 $n = 9$)	6.79 (1.91 $n = 4$)	12.92 (1.65 $n = 10$)	480 (168 $n = 4$)	4749 (1003 $n = 4$)
Frozen 15 kGy	53.22 (8.55 $n = 5$) (No test)	0.796 (0.16 $n = 16$) Yes	9.82 (3.65 $n = 6$) (No test)	18.71 (2.95 $n = 9$) (No test)	235 (111 $n = 5$) Yes	2113 (1430 $n = 5$)
Frozen 25 kGy	50.67 (3.45 $n = 8$) No	0.966 (0.08 $n = 14$) No	7.57 (1.02 $n = 8$) No	16.80 (1.27 $n = 14$) (No test)	399 (117 $n = 7$) No	4920(1430 $n = 6$)
Freeze-dried 15 kGy	18.85 (2.70 $n = 6$) Yes	0.646 (0.09 $n = 12$) Yes	3.76 (1.15 $n = 7$) Yes	21.91 ($n = 1$) No	27.2 (13.8 $n = 5$) Yes	273 (57 $n = 5$)
Freeze-dried 25 kGy	10.69 (3.97 $n = 7$) Yes	0.563 (0.13 $n = 11$) Yes	3.01 (0.94 $n = 6$) Yes	22.73 (7.83 $n = 6$) Yes	47.5 (24.3 $n = 6$) Yes	384 (215 $n = 6$)

Prior to testing, approximately 20 mm on either end of the tendons from groups 1, 2 and 3 ("wet" groups) were allowed to air dry while the main body was kept moist. Tendons from groups 4 and 5 were rehydrated for several hours before being tested, except for 20 mm on each end, which was left dry. Dry ends give rise to fewer failures at the clamp edges during testing as they tend to spread out less inside the clamp [13]. The additional effective length of a specimen with dried ends gripped in clamps similar to those of Ker [12] is near to zero (personal communication). Tendon specimens were placed with the dried ends between steel clamps in an Instron 8031 tensile testing machine. The distance from the upper clamp to the lower clamp was between 80 mm and 100 mm and was as large as the length of the specimen would allow. The displacement of the clamps, as measured by the Instron machine, was used in calculations of strain. The clamps had regular, shallow grooves, approximately 1 mm deep and 1 mm apart, running across the specimen ends which provided extra friction with the specimen. Specimens were stretched cyclically between zero and 30 MPa stress at 0.1 Hz for several cycles then stretched to failure at a rate of 0.4 mm/s. During testing all specimens were kept covered by tissue paper soaked in 0.9% saline.

The mode of failure was noted as either a break at the clamps or as a break in the middle. From the cyclic tests the slope of the stress–strain curve in the linear region (at approximately 30 MPa stress) was measured. This is the stiffness and can be called the tangent modulus. Hysteresis was also measured from the stress–strain loops from the cyclic tests as the proportion of the area between the relaxing curve and the loading curve to the total area under the loading curve [12]. The ultimate tensile stress (UTS), strain at failure, strain energy at failure per unit cross-sectional area and tangent modulus were measured from the failure tests. The strain energy at failure can be calculated from the total area underneath the stress–strain curve.

The results of each measured parameter from each group were analysed with a one-way ANOVA followed by a Dunnet's *post hoc* test with the fresh group acting as a control. Where the number of samples was too small or the data failed a normalcy test, a Kruskall-Wallis test with a Dunn's post-test with the fresh group as control were used instead. The properties of groups which were significantly different from those of the fresh group are marked with an asterisk in Table I.

3. Results

The mean values found in the tensile tests are given in Table I along with their statistical significance compared with the fresh group. Those groups significantly different from the fresh group ($p < 0.05$) are noted in Table I; those not significantly different or those upon which statistical tests could not be performed because of too few replicates are also noted. The number of replicates of different parameters within a group may be different because not all tests were successfully carried out on all specimens. The figures in brackets in Table I are the standard error followed by the number of replicates. The value given for hysteresis for the freeze-dried 15 kGy group was from a single specimen, thus no standard error is given.

The value given for the tangent modulus is taken from the cyclic tests. Fig. 1 shows the stress–strain curve of a cyclic and a failure test. This particular specimen was irradiated with 15 kGy from a γ source while frozen in water. The curve is also typical in form of the fresh specimens. The values of the tangent modulus obtained from the cyclic tests were in every case similar to 2 significant figures, and in most to 3 significant figures, to the values obtained from the failure tests.

Fig. 2a and 2b show that the freeze-dried groups were less stiff and less strong than the frozen irradiated groups at the same dose. There were no significant changes in any of the parameters between groups of similar condition (e.g. 15 kGy frozen and 25 kGy frozen), although there were trends. In all measured properties except failure strain the mean of the 25 kGy frozen group was less affected than the mean of the 15 kGy frozen group. In every case the mean of the 25 kGy freeze-dried group was further from the mean of the fresh group than the mean of the 15 kGy freeze-dried group.

Fig. 2a shows the tangent modulus of the groups and it is clear that the freeze-dried irradiated groups are less stiff than the fresh group. The stiffness of the 25 kGy frozen group is similar to that of the fresh group whereas the 15 kGy frozen group is significantly

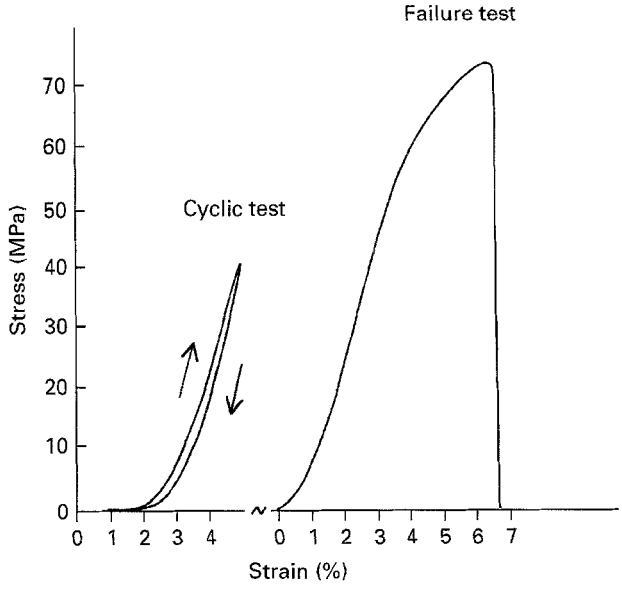

Figure 1 A typical stress–strain curve of tendon. This particular specimen had been irradiated with 15 kGy from a γ source while frozen in water. The curve is also typical for fresh specimens. The arrows indicate the direction the curve took during the test. The curve in the returning phase falls below the curve during the stretching phase because viscous energy loss results in lower forces at similar strains. The amount of energy lost, represented by the area inside the loop, can be expressed as a percentage of the total energy used in stretching the specimen, represented by the total area under the stretching phase. This is termed the hysteresis loss. A tangent can be drawn on the stretching phase in the linear region. The slope of this line is the stiffness or tangent modulus of the specimen.

less stiff than the fresh group. However the frozen 15 kGy group is less affected than either of the freeze-dried irradiated groups. Both of the frozen irradiated groups have a similar UTS to that of the fresh group, whereas the freeze-dried irradiated groups are significantly weaker. This pattern is also reflected in the results of failure strain (Fig. 2c), hysteresis (Fig. 2d) and strain energy at failure (Fig. 2e and f). The strain energy required to failure is expressed in terms of both the cross-sectional area (e) and the volume (f) of material under stretch.

In general the freeze-dried irradiated groups were affected more than the frozen irradiated groups. There is a non-significant trend for the higher dose frozen irradiated group to be less severely affected than the lower dose group and for the higher dose freeze-dried irradiated group to be more severely affected than the lower dose group. The 25 kGy frozen group changed the least of all.

The values of parameters measured in the failure tests do not include data from specimens which ruptured at the clamp edge, i.e. UTS, failure strain and strain energy at failure. However, the inclusion of the data from clamp edge ruptures would not alter the pattern of differences between the groups.

4. Discussion

The pattern of changing mechanical properties seen here among the dry and wet irradiated groups can be

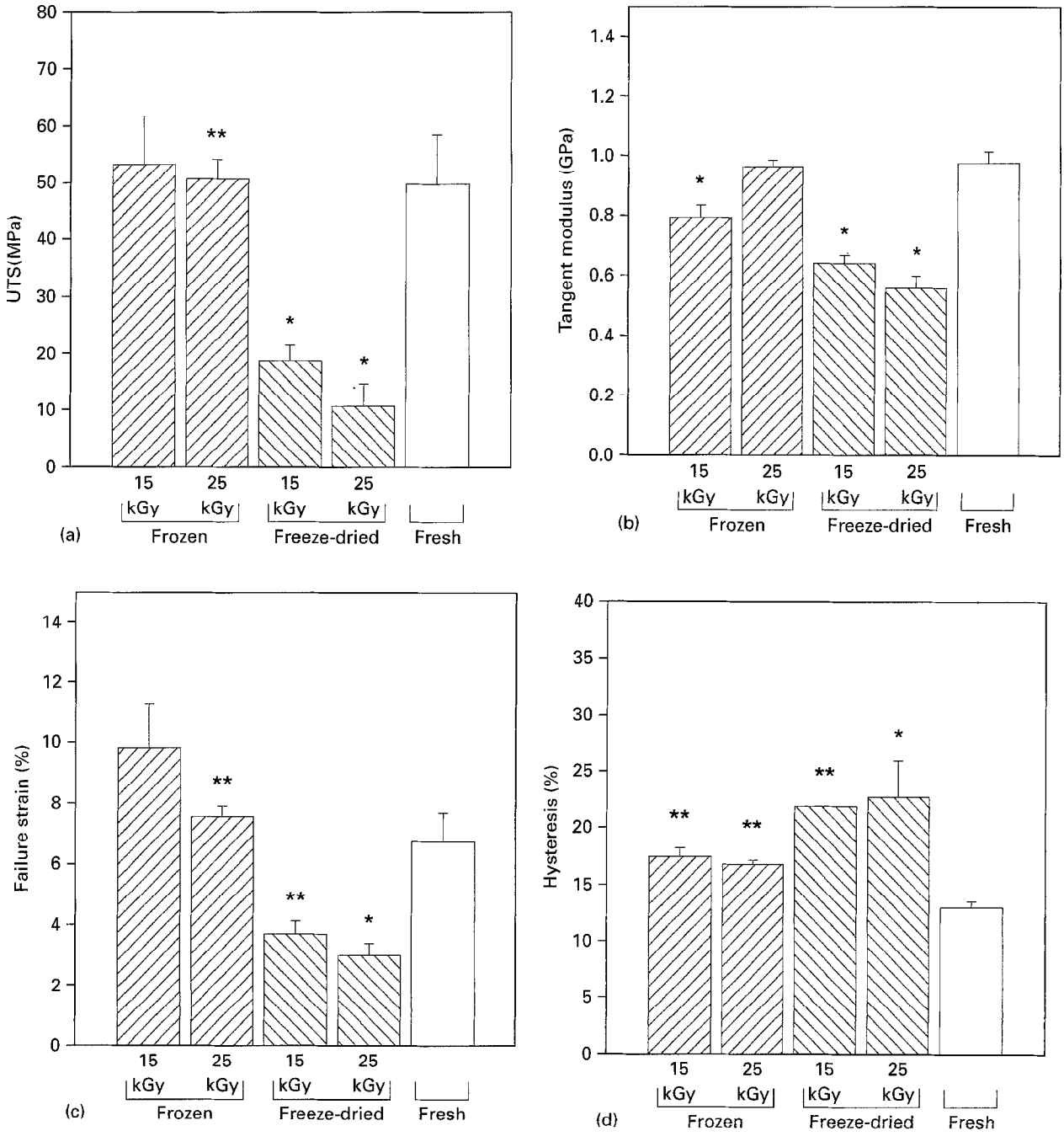

Figure 2 (a) The mean values of the UTS of the five groups. The error bar shows the standard error. Groups significantly different from the fresh group are marked with an asterisk. (b) The mean tangent modulus (stiffness) E of the groups, measured from the cyclic tests. (c) The mean strain at failure of the five groups. (d) The mean hysteresis loss during cyclic stretching. The value for the freeze-dried 15 kGy group is from one sample. Groups with small sample numbers which could not be statistically tested are marked with a double asterisk. (e) The mean strain energy at failure, expressed per unit cross-sectional area and (f) per unit volume.

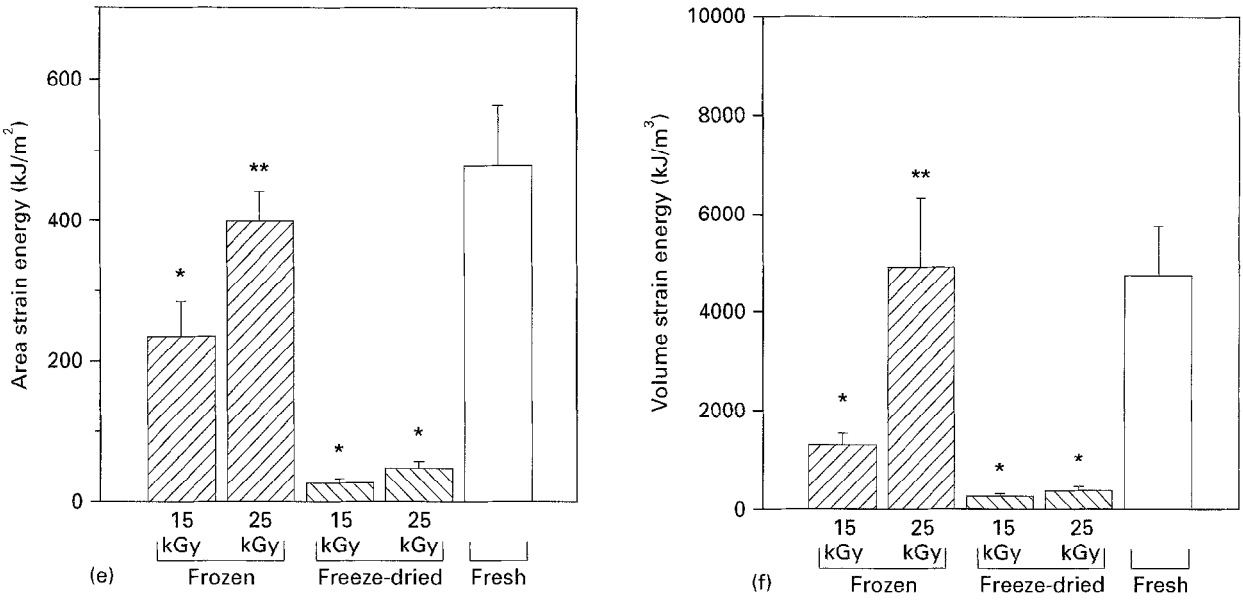

Figure 2 (Continued)

TABLE II A table of the statistical significance of the differences between groups of similar conditions and doses. The p values of each test between groups is given along with the kind of test used- 't' indicates a t-test, 'M' indicates a Mann–Whitney U-test ranked sum, NT indicates no test was used (because $n = 1$). A Mann–Whitney U-test was used where the data did not pass a normalcy test which is required for a t-test.

Parameter	Frozen 15 versus Frozen 25		Freeze-dried 15 versus Freeze-dried 25		Frozen 15 versus Freeze-dried 25		Frozen 25 versus Freeze-dried 25	
UTS	0.4600	t	0.0027	t	< 0.0001	t	0.0027	t
E	0.0013	t	0.0984		< 0.0001	t	0.0984	t
Strain	0.0814	M	0.2624	t	< 0.0001	t	0.2624	t
Hysteresis	0.0950	M	NT		0.0035	M	NT	
Energy (kJ/m^2)	0.0348	t	0.0823	M	< 0.0001	t	0.0823	M
Energy (kJ/m^3)	< 0.0001	t	0.4848	M	0.0007	M	0.4848	M

explained with the mechanisms put forward by Bailey and co-workers [6–8]. These results confirm that irradiation of collagen without water present results in a greater degree of mechanical damage than if water were present. An increased dose in the freeze-dried groups lead to a greater impairment of the mechanical properties, suggesting that there were more scission events along the collagen α chains. Whereas, the frozen irradiated group suffered no change in properties, suggesting that crosslinking adequately compensated for α chain scission.

If we were to consider collagen at the ultrastructural level the tropocollagen macromolecules and their surrounding proteoglycan/water matrix might be considered to be a short parallel fibred composite. The effect of scission of the tropocollagen α chains would be to weaken and shorten the fibres of the material. This would lead to a material of lower strength and lower tangent modulus [17], as was so in the freeze-dried irradiated groups. If, however, water is present during irradiation extra intramolecular cross links are formed, either by free radicals or chain mobility [8]. The effect of this would be to join up cut α chains thereby reversing somewhat the weakening effect and the fibre shortening effect of chain scission. This would allow load transfer along the complete length of the collagen molecules, across newly formed intramolecular crosslinks. This would explain the increased strength of the higher dose frozen irradiated group. Bailey and co-workers [8] have observed a similar effect in the maximum tensile load of wet and dry irradiated rat tail tendons; wet irradiated being stronger than dry irradiated.

These tests have shown that the mechanical consequences of irradiation of collagenous tissue are adequately explained by the mechanisms proposed by Bailey and co-workers. If irradiation is to be used as a sterilization technique for tendon grafts with intended mechanical functions, it should be performed while water is present with the collagen. Clearly, irradiation of dried collagen cannot be satisfactorily used with load-bearing grafts, such as an anterior cruciate ligament replacement.

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