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The effect of sterilization and storage on the viscoelastic properties of human tendon allografts

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ABSTRACT

Allografts have become increasingly preferred for anterior cruciate ligament replacement purposes. The risk of infections necessitates thorough sterilization procedures, and the allografts usually need to be stored prior to surgery. Classical mechanical tests have been performed with various types of tendons, however, tibialis anterior and peroneus longus tend to suffer the least biomechanical changes after irradiation. Only few results are available of the strain and creep behaviour of tendons, even though this information is necessary to provide suitable allografts. The aim of the present study is to analyze the effect of different tendon types (T-tibialis anterior, P-peroneus longus), sterilization methods (G-gamma irradiation of 21 kGy, E-electron beam irradiation of 21 kGy) and storage times (5 and 6 months) on the creep behavior, which is characterized by the strain at the end of the loading phase and creep deformation after static loading. Static creep tests were performed with 250 N load during 60 s. Deformation at the end of the loading phase of both tendons was significantly smaller after 5 months long storage than that after 6 months long storage. TE5 showed significantly less creep than group TE6, and TE6 significantly greater than PE6. The creep of TE5 was significantly lower than that of TG5. Based on the data, the peroneus longus sterilized by electron beam and stored deep frozen for 5 months is a better choice for anterior cruciate ligament reconstruction than tibialis anterior sterilized by gamma irradiation stored for 6 months.

1. Introduction

Donor-derived tendons, also known as allografts, are increasingly utilized for replacement purposes due to their numerous benefits, especially for anterior cruciate ligament (ACL) reconstruction. Using allografts, shorter operating times, less postoperative pain, no donor-site morbidity and smaller surgical scars can be expected [4,5,10,18,23,24,36]. On the other hand, allografts can transmit bacterial and/ or viral infections. Therefore, allografts should be sterilized before implantation. However, it is impossible to sterilize without compromising the biomechanical properties of the tissues. [3] Several sterilization techniques exist, including chemical sterilization methods, different types of ionizing radiation and combined methods. Two of the most used ones are gamma irradiation and electron beam irradiation, as they are simple, safe and energy efficient processes. [34] Their effectiveness in sterilization lies in their ability to easily penetrate the inside of the material and inactivate microorganisms without heat exchange problems, pressure differences or diffusion barriers. The radiation allows the sterilization of heat-sensitive biological materials and is effective at room temperature and even below 0 °C. [3,9] In order to avoid side effects and protect the tissues, radio-protection via combined crosslinking and free radical scavenging is usually used. The radioprotectant solutions do not introduce unnecessary chemicals to the tendons and do not change their biomechanical parameters. [14,17,33,34]

Before surgery, allografts often need to be stored in a safe way. Deep freezing is the simplest, most economical, and most widely used method. [3,5] According to previous studies, freezing has relatively little effect

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on the structural and mechanical properties of soft tissues. [6,8,25] Tissue banks recommend a wide range of allograft types for ACL replacement purposes. The most common ones are fascia lata, tibialis anterior or posterior, femoral tendons, Achilles tendon, and bone-patella tendon-bone allografts [5,10,15,23,29]. Our previous experiments [17] and the data of the international literature [2,27] show that the tibialis anterior and peroneus longus tendons tend to suffer the least biomechanical changes after irradiation. Comparing groups sterilized by 21 kGy of gamma irradiation, the Achilles showed significantly lower Young modulus of elasticity than the peroneus longus ($p = 0.028$) and tibialis anterior ($p = 0.001$). Similarly, in case of sterilization by 42 kGy of gamma irradiation the Achilles reached significantly lower values of Young modulus of elasticity than the peroneus longus ($p = 0.000042$) and tibialis anterior ($p = 0.00142$). Therefore, we continued the examinations with only these two types. The dose of 42 kGy affected the biomechanical properties in most graft types, that is why we focus on the bactericide dose (21 kGy) rather than the virucide dose (42 kGy). [17,21]

Grafts should have structural properties similar to those of the native ACL. The most studied characteristics of allografts and autografts are the Young modulus of elasticity, maximum force, stiffness, relative elongation, and elongation at break [1,2,7,17,18,22,27]. It is important for allografts to be flexible to allow natural movement [20], however, it is not advisable to choose tendons for knee ligament reconstruction surgeries that respond to mechanical stress with high elongation, as the joint may lose its stability and cannot provide adequate resistance to the forces applied to it, this is why creep tests are necessary as well [26]. The purpose of the present study was to biomechanically evaluate with a load test the creep and deformation in two types of tendon allografts used in ACL reconstruction – peroneus longus and tibialis anterior –, caused by different sterilization techniques – gamma irradiation and electron beam irradiation – and storage times – 5 months and 6 months –. Soft tissues are usually used for surgeries for up to 6 months, it is rare in the common practice to store them for more time. Thus, we examined the last two months of the 0–6 months period in favor of safety. [35] We hypothesize that the results of the present research can provide help in choosing the most optimal techniques for storing and sterilizing tendon allografts.

2. Materials and methods

Our study included 60 peroneus longus and 60 tibialis anterior tendons collected from human cadavers using sterile surgical protocols, within 24 h post-mortem. Due to undesirable forms of failure, only 43 tibialis anterior and 42 peroneus longus tendons' measurements could be evaluated. Sufficient tendons were tested in each category to draw conclusions from the measurements.

The soft tissues surrounding the tendons were removed, then the allografts were screened for degenerative lesions and viral infections, resulting negative. Based on medical history, none of the donors had previously tendon related injuries or illnesses. Protection of tissue properties against ionizing radiation using radio-protective treatment scavenging free radicals have been suggested for allografts in order to prevent radiation-induced decreases in mechanical strength. [30,34] Numerous experiments have been carreid out to find the most optimal method of radio-protection which can possibly be used by tissue banks as well in the near future. [31,32,33] A study written by Grieb et al. [14] suggested that following a pretreatment with a radio-protectant solution high dose of gamma irradiation can reduce infectious risks associated with soft tissue allografts while maintaining the original biomechanical performance of the tissues. The samples were replaced immediately in a premixed radio-protectant solution containing 16.7% 1,2-propanediol, 24.2% dimethyl-sulfoxide, 3.8% D-trehalose, 2.7% D-mannitol all w/w (Sigma-Aldrich, Saint Louis, USA). [14,17] Afterwards, each tendon was deep frozen at −70 °C.

Groups were created according to the storage time, type of irradiation and the type of the graft. The name of each group consists of three characters, first of which is the type (T - tibialis anterior, P - peroneus longus), the second is the mode of sterilization (G - low dose gamma irradiation, E - low dose of electron beam irradiation), and the third indicates the duration of storage by deep freezing (5–5 months, 6–6 months). Originally 4 more groups were tested: TF5, TF6, PF5, PF6 (F - deep frozen only). TF5 and TF6 were tested in a different manner than all the other groups, so the results were not comparable to the other groups. PF5 and PF6 did not show any significant differences either, and allografts without sterilization could not be usen for surgeries anyways, so we have decided to leave these out of the evaluation.

Tendinous tissues respond to stresses in a viscoelastic manner, thus, they deform in a non-linear manner in response to the applied loading characteristics. The deformation develops in a time dependant manner. [28] A creep test can be used to verify the response of a given sample to a constant mechanical tensile load. The basis of the tests is that with special loading devices the test sample is subjected to a constant load for a given period of time after a rapid loading phase, while the measurement data, in this case forces and displacements are continuously registered. [37] Over time, the material changes according to its mechanical properties, deformation or changes in orientation can occur. From the results of a creep test of only a few minutes, the expected deformation can be inferred over a longer period of time. In the current study, measurements were performed with an Instron 8872 servohydraulic load frame (Instron Ltd., High Wycombe, UK) equipped with a 25 kN load capacity Instron Dynacell load cell, an Instron Fasttrack 8800 control unit and a freezer clamp structure at the accredited materials testing laboratory of the Budapest University of Technology and Economics Biomechanical Research Centre. The tests were authorized by the Research Ethics Committee of Uzsoki utcai and Péterffy Sándor Hospital (number: 03/2009).

Before the measurements, the grafts were thawed on the day of the test at room temperature and then at 37 °C for 20 min immediately before the test. The free ends of each graft were sewn together before the measurement, so doubled tendons were tested. The samples were then clamped and connected to the material testing machine as shown in Fig. 1. The distance between the clamps, the measuring length was

Fig. 1. Measurement arrangement: One end of the allograft stabilized by clamps and dry ice, the other end by a metal shaft.

exactly 60 mm in each case. The clamp was frozen in dry ice to provide stable fixation. [16] In order to avoid re-freezing of the tissues, freezing lasted only 3 min. The following parameters were set in the control program, the Bluehill and Single Axis MAX software: first the samples were preloaded at a speed of 20 mm/min up to 2 N, then loaded at a speed of 50 mm/min up to 250 N. After reaching 250 N, the samples were kept under load for 60 s.

In order to compare the biomechanical properties of the specimens, we used the following two parameters: strain at the end of the loading phase and creep deformation. Strain is the deformation that solid objects undergo due to external mechanical loads. It is defined as the difference between the dimensions of the object before and after loading, the linear part seen at the beginning of the curve diagrams (X1 in Fig. 2.). The strain during this time corresponds to the momentary elastic deformation component of the deformation. The duration of this stage varies from sample to sample. From a practical point of view, although it is important for the graft to have a flexible deformation component that allows suturing and natural movement, it is not advisable to choose tendons for knee ligament reconstruction surgeries that respond to mechanical stress with high elongation, as the joint may lose its stability and cannot provide adequate resistance to the forces applied to it.

Creep deformation is defined as the difference in the measured lengths at the end of the loading phase and after 60 s of static loading, the nearly horizontal part of the creep curves (deformation between X1 and X2 in Fig. 2.). It corresponds to the combined effect of the delayed elastic strain and the viscous flow components. The length of the ideal allograft does not change over time due to constant mechanical stress, thus ensuring that the joint restored during surgery will perform its role properly in the long run. In reality, due to the phenomenon of creep, this is not feasible, so the aim is to find an allograft with the least possible deformation.

2.1. Statistical analysis

Statistical analysis was performed with the StatSoft Statistica 13.3 (StatSoft Inc., Tulsa, OK, USA) software. Data were presented for each group as a median with the corresponding interquartile range (25% and 75% percentile). The groups were compared according to graft type, sterilization method, and storage time. Values measured during biomechanical studies often follow a normal distribution, but for small sample sizes this condition is not met in every case. As the real mean, standard deviation, and distribution of the examined parameters of each group are not known, we used the non-parametric Mann-Whitney *U* test to compare the categories (separately by graft type, sterilization method, and storage time). It tests the null hypothesis that the distribution of the dependent variable is the same for the two groups and therefore from the same population. In all studies, a p-value of less than 0.05 was considered statistically significant. [11]

3. Results

The resulting strain–time curves of each group represented by their medians are shown in Fig. 3. The numerical data is summarized in Tables 1–2. Figs. 4-5 show the median, maximum, and minimum values, as well as the interquartile ranges for the two parameters investigated. The effects of different storage times are as follows: The deformation at the end of the loading phase of group TG5 was significantly smaller than that of group TG6 ($p = 0.0375$) and group PG5 elongated significantly less during the loading phase than group PG6 ($p = 0.0122$), as shown in Table 1 and Fig. 4. Group TE5 responded to the same load with significantly less creep deformation than group TE6 ($p = 0.0257$), as shown in Table 2 and Fig. 5. In every further pairing (strain of TE5-TE6, PE5-PE6, creep of TG5-TG6, PG5-PG6, PE5-PE6), the program calculated a significance level of $p > 0.05$, therefore by accepting the original null hypothesis, it can be stated that there is no significant difference between them .Table 3 and Table 4.

The effects of different allograft types are as follows: Creep deformation suffered by group TE6 was found to be significantly greater than that of group PE6 ($p = 0.0414$), as shown in Table 2 and Fig. 5. In other cases (strain of TG5-PG5, TG6-PG6, TE5-PE5, TE6-PE6, creep of TG5-PG5, TG6-PG6, TE5-PE5) there were no significant difference found between the two graft types based on the deformation at the end of the loading phase or the creep deformation values (Tables 3–4).

The effects of different sterilization methods are as follows: The creep deformation value of group TE5 was significantly lower than that of group TG5 ($p = 0.0414$), as shown in Table 2 and in Fig. 5. In any other pairing (strain of TE6-TG6, PE5-PG5, PE6-PG6, creep of TE5-TG5, TE6-PG6, PE5-PG5, PE6-PG6), the differences are not statistically significant (Tables 3–4).

4. Discussion

Nowadays, primarily autolougus tendons are the gold-standards for ACL reconstructions, but in case of revision or multiple ligament injuries it is more appropriate to use donor-derived tendons instead of the patient’s own tissues. The type of the graft, the method of treatment, and the duration of storage can all influence the biomechanical properties of the grafts to different extent. [3] The aim of the current study was to evaluate the differences of the effects of different tendon types – peroneus longus and tibialis anterior –, sterilization methods – gamma

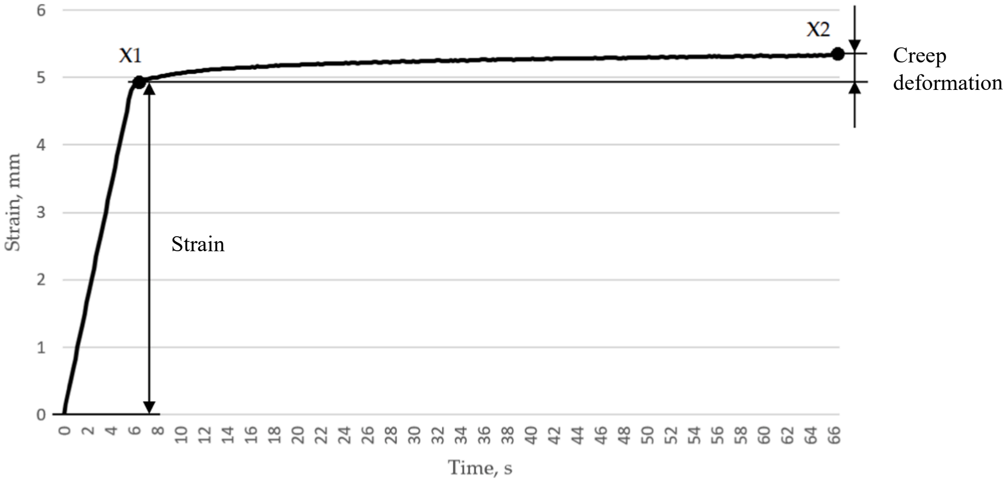

Fig. 2. Example for defining parameters: The strain at the end of the loading phase is defined by X1, the creep deformation is defined as the difference between X2 and X1.

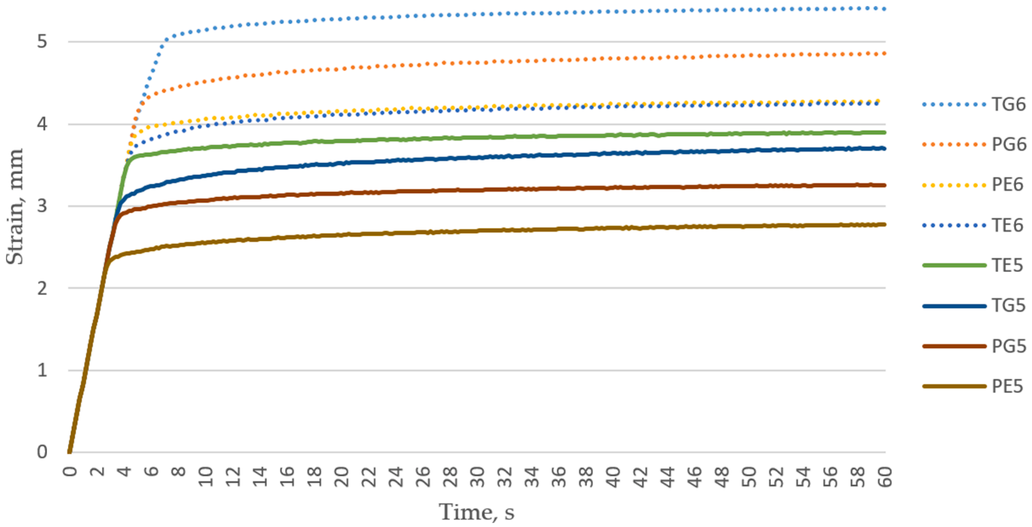

Fig. 3. Creep curves as a function of time recorded during the static tests of the 8 groups of tendons.

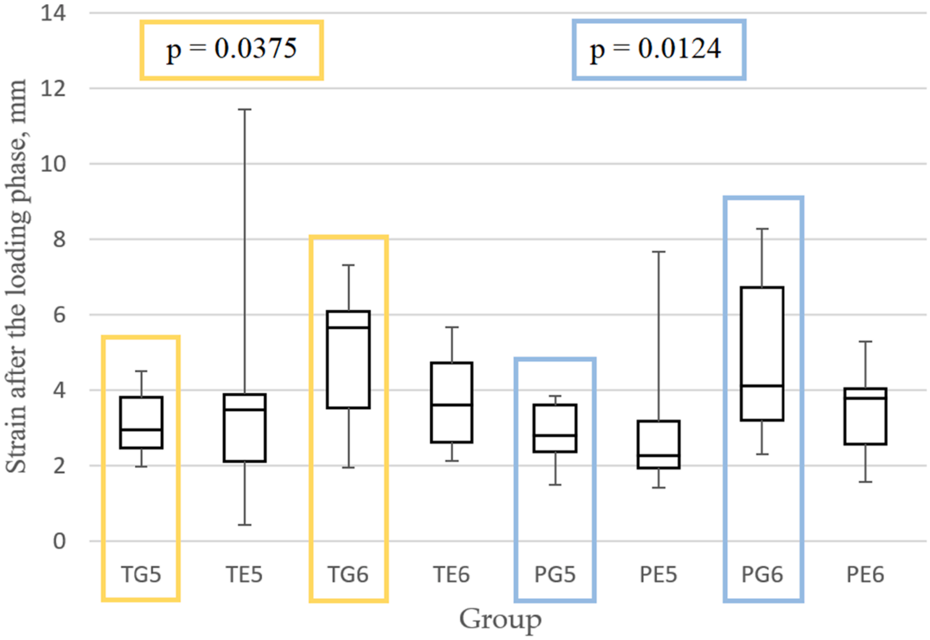

Fig. 4. Values of strain at the end of the loading phase to 250 N. Median, 25 and 75% percentile, minimum and maximum values are illustrated. – significant differences are signed with colors, and the corresponding p-values are written.

irradiation and electron beam irradiation –, and storage times – 5 and 6 months – on the strain and creep behaviour of tendon allografts in order to find the parameters allowing the least deformation, making the tendons suitable for ACL reconstruction.

On the basis of the statistical analysis of the measured data, the following can be established: the peroneus longus type is recommended over tibialis anterior, sterilization by electron beam irradiation is recommended over gamma irradiation, and deep freezing for only 5 months is recommended over 6 months. Groups with these parameters suffered less deformation during both the load increasing period and static loading, making them more suitable for knee ligament reconstruction surgeries. The ratios of creep deformation and strain are shown in Fig. 6. It is clearly visible that more storage time has a greater effect on strain than on creep deformation.

Firstly, we compared the samples according to their storage times. As soft tissues are usually used for surgeries in common practice for up to 6 months, we examined the last two months of the 0–6 months period in favor of safety. [35] We paired the groups so that only the storage time differed between two groups, the graft type and the treatment method were the same. The tests thus indicated that the amount of creep deformation also increases with increasing storage time (Fig. 5). The same relationship is true for the deformation at the end of the loading phase (Fig. 4). The deformation at the end of the loading phase of group TG5 was significantly smaller than that of group TG6 ($p = 0.0375$) and group PG5 elongated significantly less during the loading phase than group PG6 ($p = 0.0122$). Group TE5 responded to the same load with significantly less creep deformation than group TE6 ($p = 0.0257$). Giannini et al. [12] examined the effects of storage by deep freezing at −80 °C on the histological and structural properties of the human posterior tibial tendon. Compared to non-frozen controls they found an increase in the mean diameter of collagen fibrils and in fibril non-occupation mean ratio, while the mean number of fibrils decreased, which can explain the changes of biomechanical properties experienced in our study. [12]

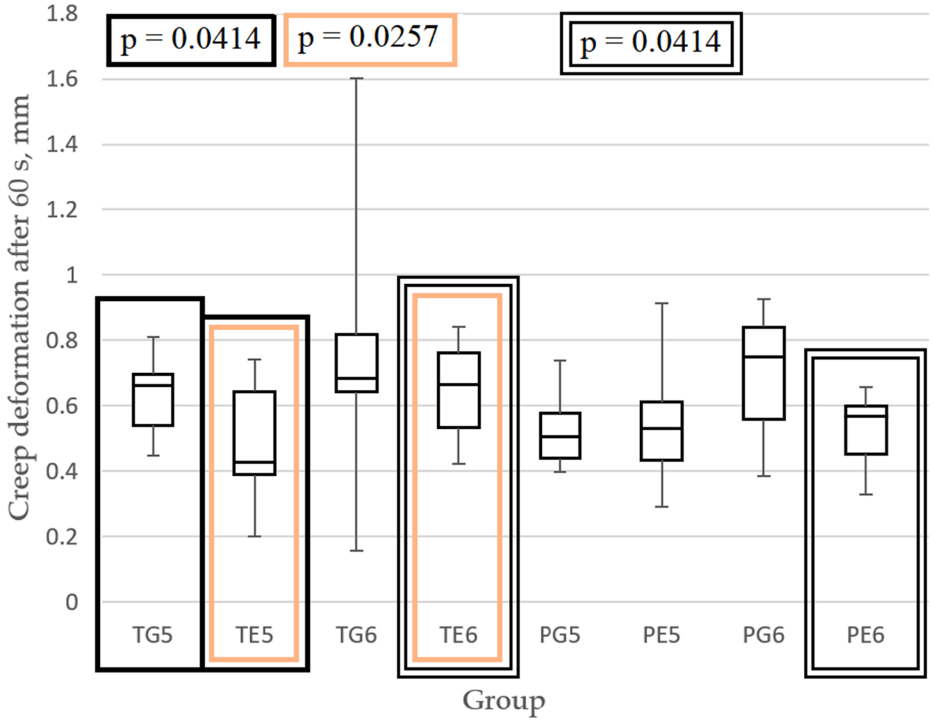

Fig. 5. Values of creep deformation after 60 s of static load of 250 N. Median, 25 and 75% percentile, minimum and maximum values are illustrated. – significant differences are signed with colors and lines, and the corresponding p-values are written.

Table 1
Numerical results of strain at the end of the loading phase (difference between the length of the allografts before and after loading), mm – the colors show the pairs of groups with significant difference.

Number of samples	TG5	TE5	TG6	TE6	PG5	PE5	PG6	PE6
1.	3.7977	3.4777	5.9573	4.9946	2.5989	7.6589	8.2713	5.28220
2.	3.4763	2.2454	6.1372	2.6096	3.4864	2.8337	5.9577	4.1368
3.	4.1488	0.4137	5.6630	3.6002	2.7599	2.2690	2.8093	2.4884
4.	2.9426	3.6110	2.6566	3.8352	3.6326	1.7801	2.2911	3.8236
5.	2.1258	1.9878	1.9320	2.1611	3.6387	2.2667	4.1209	1.5493
6.	2.4436	3.6270	3.4435	2.1225	1.4850	1.4104	3.4358	2.6465
7.	1.9541	4.1389	3.8178	2.6224	3.8288	3.6368	2.9643	3.9463
8.	3.7917	5.2795	6.9435	2.9876	1.6306	3.2781	7.9920	3.1649
9.	4.4981	2.6696	5.6491	4.6004	2.8081	1.9260	3.9989	4.1518
10.	2.6637	11.4503	7.3061	4.8136	2.2844	1.9546	7.4714	2.2964
11.	2.4699	0.6045	–	5.6626	–	–	5.1499	3.7846
Median	2.9426	3.4777	5.6561	3.6002	2.7840	2.2678	4.1209	3.7846
Lower quartile	2.4568	2.1166	3.5371	2.6160	2.3630	1.9332	3.2000	2.5675
Upper quartile	3.7947	3.8830	6.0922	4.7070	3.5961	3.1670	6.7145	4.0415

Table 2
Numerical results of creep deformation (difference between the measured lengths at the end of the loading phase and after 60 s of static loading of 250 N), mm – the colors and lines show the pairs of groups with significant difference.

Number of samples	TG5	TE5	TG6	TE6	PG5	PE5	PG6	PE6
1.	0.6987	0.4283	0.6937	0.75941	0.3968	0.6204	0.8419	0.5923
2.	0.4473	0.4690	0.8418	0.47283	0.7010	0.8463	0.9250	0.6037
3.	0.6617	0.2047	0.5109	0.66446	0.4320	0.4934	0.3851	0.3376
4.	0.7736	0.7423	0.7478	0.61761	0.7398	0.9136	0.4057	0.4715
5.	0.6960	0.7162	0.1578	0.67928	0.5770	0.5199	0.7513	0.3293
6.	0.5105	0.6148	0.6412	0.56245	0.5747	0.3557	0.7994	0.5957
7.	0.5722	0.6731	0.8413	0.76406	0.4881	0.5830	0.8409	0.5692
8.	0.6339	0.3868	0.6451	0.42367	0.4361	0.5395	0.8716	0.6124
9.	0.6779	0.3931	1.6026	0.50561	0.5265	0.2908	0.5848	0.6590
10.	0.8095	0.4150	0.6739	0.84315	0.4476	0.4163	0.5823	0.4365
11.	0.4884	0.2016	–	0.78550	–	–	0.5334	0.4974
Median	0.6617	0.4283	0.6838	0.6645	0.5073	0.5297	0.7513	0.5692
Lower quartile	0.5413	0.3900	0.6421	0.5340	0.4389	0.4356	0.5578	0.4540
Upper quartile	0.6973	0.6440	0.8179	0.7617	0.5764	0.6110	0.8414	0.5997

Table 3
p-values of the strain at the end of the loading phase of the paired groups – p-values less than 0.05 are signed green.

	TG5	TE5	TG6	TE6	PG5	PE5	PG6	PE6
TG5		1.0000	0.0375		0.5485			
TE5	1.0000			0.5552		0.4593		
TG6	0.0375			0.0836			0.9681	
TE6		0.5552	0.0836					0.7949
PG5	0.5485					0.4715	0.0124	
PE5		0.4593			0.4715			0.0836
PG6			0.9681		0.0124			0.1310
PE6				0.7949		0.0836	0.1310	

Table 4
p-values of the creep values of the paired groups – p-values less than 0,05 are signed green.

	TG5	TE5	TG6	TE6	PG5	PE5	PG6	PE6
TG5		0.0414	0.3789		0.0989			
TE5	0.0414			0.0257		0.4179		
TG6	0.3789			0.5485			0.9681	
TE6		0.0257	0.5485					0.0414
PG5	0.0989					0.8493	0.0524	
PE5		0.4179			0.8493			0.9681
PG6			0.9681		0.0524			0.0873
PE6				0.0414		0.9681	0.0873	

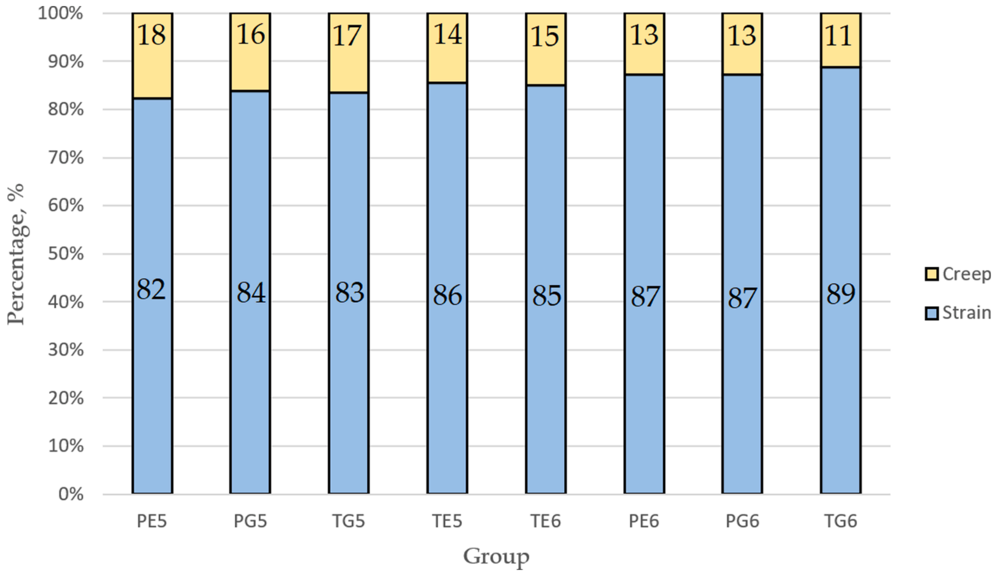

Fig. 6. Percentages of creep deformation and strain at the end of the loading phase – the numerical data is written on the diagram.

Secondly, for the comparison of allograft types we made pairs of groups only differing in type. We found that peroneus longus tendons are less prone to creep deformation than tibialis anterior tendons (Table 2, Fig. 5). Creep deformation suffered by group TE6 was found to be significantly greater than that of group PE6 ($p = 0.0414$). In a previous study [17] carried out in our laboratory Achilles, quadriceps, semitendinosus + gracilis, tibialis anterior and peroneus longus tendons were compared in three groups: non-irradiated, gamma irradiated with a dose of 21 kGy, and gamma irradiatied with a dose of 42 kGy. To compare the biomechanical properties of the specimens, the following four parameters were used: Young modulus of elasticity, maximum load, strain at tensile strength and strain at rupture. Cyclic loading tests were performed followed by load to failure tests. The doubled tibialis anterior and peroneus longus tendons performed the best results among the other described ACL grafts.

Thirdly, the grafts were also compared for the two parameters according to the two sterilization methods. The groups compared differed only in the mode of treatment, while the tendon type and the storage period were the same. The biomechanical changes created by gamma irradiation resulted in greater creep deformation values than those created by electron beam irradiation (Table 2, Fig. 5). The creep deformation value of group TE5 was significantly lower than that of group TG5 ($p = 0.0414$).

The effects of ionizing radiation have been examined on other important parameters – such as maximum load, Young's modulus of elasticity, strain at tensile strength and toughness – than creep and strain previously. [17,19,31] Kamiński et al. [19] did not find a difference between the effects of different doses (0–100 kGy) of electron beam irradiation during an experiment with human bone-patellar tendon-bone grafts. [19] In contrast, Seto et al. [31] did report a decrease in maximum load, Young's modulus of elasticity, strain at tensile strength and toughness as a result of low dose (25 kGy) andhigh dose (50 kGy) gamma irradiation, as well as electron beam irradiation on rabbit tendons. [31] In the study carried out by Hangody G. et al. (2017) it was found that gamma irradiation even in low dose (21 kGy) caused an increase in Young's modulus of elasticity, maximum load and strain at tensile strength, as well as a decrease in strain at break compared to non-irradiated control groups in case of both penoneus longus and tibialis anterior tendons [17]. These findings support our results stating that electron beam irradiation is a better option than gamma irradiation.

There are certain limitations to this study that have to be mentioned. In the present experiment the effects of storage time was examined only for the 5 and 6 months periods. In the future, additional measurements will be neccessary to study changes caused by storage during 0, 1, 2, 3, and 4 months after tissue extraction. It is also worthwhile to investigate another characteristic behavior of viscoelastic materials, such as ligaments and tendons: stress relaxation. In order to do this, instead of a static load the tissues must be subjected to a constant amount of deformation while the decreasing tendency of mechanical stress must be registered by a software. As the range of motion of the joints is typically limited,these conditions also occur during everyday life events. [13] The current study revealed that considering the creep and strain behaviour, the recommended parameters for allografts used in ACL reconstruction are peroneus longus over tibialis anterior, electron beam irradiation over gamma irradiation and only 5 months of storage over 6 months.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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