Good preservation of initial mechanical properties in lipid-extracted, disinfected, freeze-dried sheep patellar tendon grafts

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ABSTRACT **Patellar tendon allografts can be used for anterior cruciate reconstruction to avoid morbidity of autografts on the donor side. Secondary processing of allografts is important for reducing immunological reactions, bacterial contamination and improving storage. We analyzed the effects of processing on the mechanical properties of patellar tendon grafts in 20 sheep. Group I (n = 10) was deep-frozen at –80 °C. Group II (n = 10) was processed by a lipid extraction/freeze-drying method, including iodoacetic acid disinfection. The contralateral tendons, freeze-dried by dehydration in a vacuum at –50 °C for 3 hours, served as controls. We measured failure stress: group I (53, SD 14 MPa), control (26, SD 15 MPa) (p = 0.04); group II (49, SD 13 MPa), control (28, SD 5 MPa) (p = 0.08). Failure strain, normalized stiffness, and energy to failure were similar in the groups. Our method of extended processing did not change the properties of the deep-frozen patellar tendons. Therefore in vivo experiments can be used when studying the effects of transplantation on mechanical properties.**

Anterior cruciate ligament deficiency can be treated by autogenic ligament or tendon grafts, but in some cases, local graft excision causes complications (Christen and Jakob 1992). Shino et al. (1990) and Collette et al. (1991) reported good clinical results with allogenic transplants. However, immune reactions have occurred that were related to tendon tissue cells (Minami et al. 1982, Pinkowski et al. 1989, Jackson et al. 1990). Further processing of bone allografts by freeze-drying or lipid extraction reduces the immune response (Friedlaender et al. 1984, Thorén et al. 1995). Previous studies on the processing of soft tissue transplants have been carried out in different conditions—e.g., freezing or freeze-drying (Thomas and Gresham 1963, Turner et al. 1988, Bechthold et al. 1994, Silver et al. 2000), disinfection with solvent agents (Maeda et al. 1993, Zimmerman et al. 1994, Burd et al. 2000) or sterilization (Bechthold et al. 1994, Jackson et al. 1990, Johnson et al. 1999). None of these methods meets all the requirements for a strong, safe and minimally-immunogenic transplant and they are therefore not recommended by the European Association of Tissue Banks (EATB 1997). We based the present study on a lipid extraction, decontamination and freeze-drying procedure that was first used only in bone grafts (Urist et al. 1975, Prolo et al. 1982). For use in tendons, we had to modify the method and omit the application of hydrochloric acid 0.6N. This method was chosen because excellent bone remodeling has been reported, relating to the preservation of bone morphogenic protein (BMP) (Thorén et al. 1995). Our hypothesis was that this processing method would not destroy the mechanical properties of frozen soft tissue transplants.

Animals and methods

The study was done in a cadaver sheep model. 20 young animals were selected from a homogeneous

Table 1. Dimensions of patellar tendons. Values are given as mean (SD)

	Group I Deep-frozen	Control Freeze-drying	Group II Lipid extraction, disinfection, freeze-drying	Control Freeze-drying
Length (mm)	49 (3)	50 (4)	51 (2)	55 (4)
Area (mm^2)	30 (5)	35 (5)	32 (5)	37 (6)

group in the abattoir, having an average age of 18 (17–19) months. Their average weight was 39.6 (30–44) kg. Before the experiment, approval was obtained from an Institutional Review Board. The patellar tendon was chosen because it has good initial mechanical properties and is used clinically as a graft for anterior cruciate ligament deficiencies (Jackson et al. 1990, Shino et al. 1990). 20 pairs of knees were randomized and placed in two experimental groups. The contralateral knees served as matched controls (Table 1). In group I (n = 10), deep frozen specimens were compared to the freeze-dried controls. We used freeze-drying in the controls because it is the commonest method for soft tissue storage in large national tissue banks and permits further extended processing; deep freezing is used more in smaller units (EATB 1997, Delloye et al. 1991). In group II (n = 10), the effect of lipid extraction/disinfection/freeze-drying was compared to freeze-dried controls (Table 1).

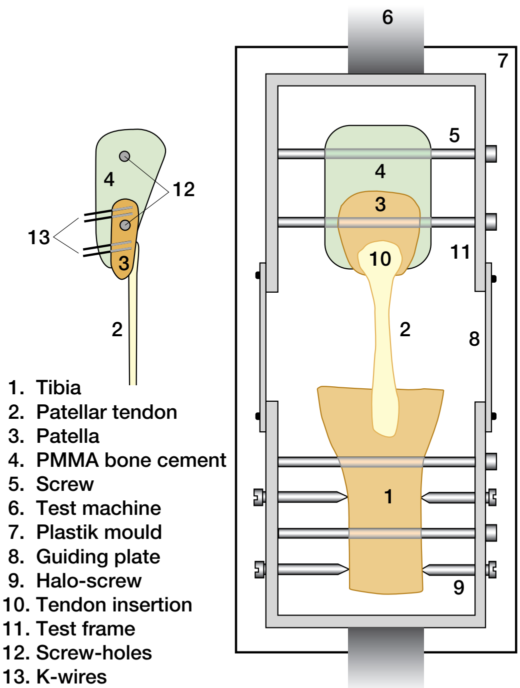
Preparation and processing

Before preparation and testing, the extremities were amputated 10 cm above the knee joint and stored for 24 hours at 4 °C. We first dissected all soft tissues including muscles, capsule, ligaments and menisci. Then the patellar tendon was prepared at room temperature (21 °C) within 30 minutes. During this procedure and the subsequent mechanical testing, the tendon was kept moist by dipping it in 0.9 % NaCl. Group I specimens were stored deep frozen at –80 °C and sealed under vacuum in paper-peel packs.

All specimens in the control groups and group II were washed in Ringer's solution for 2 hours at 22 °C and then rinsed in distilled water for 10 minutes at 22 °C. The additional lipid extraction procedure was done only in group II for 24 hours at +4 °C, using the method described by Prolo et al. (1982) and Urist et al. (1975). Washing was done by bathing the specimens in a 1:1 mixture of chloroform and methanol, which was changed twice every 24 hours. The decontamination procedure was then done for 3 days, using 10 mMol iodoacetic acid. During the procedure, the NaCl was phosphate-buffered (pH 7.5) and sodium azide was added to stop enzymatic activity against the bone morphogenetic protein (BMP) by sulfhydril group inhibitors (Prolo et al. 1982). Freeze-drying was performed in the control groups and group II after they had been placed in a vacuum of 0.3 mbar for 12 hours at 22 °C. All specimens were placed unfrozen in the lyophilizer (model 12; Virtis Freezemobil, Germany). This procedure was done in a vacuum of 1 mbar with a maximal temperature of +31.2 °C and a condenser temperature of –50 °C for 72 hours. The efficiency of dehydration was measured by weight and magnetic resonance (Magnetom 1.5 Tesla), which confirmed a residual water content of less than 5% of the initial values.

Geometry of the specimens

The patellar tendon insertion was regarded as being in the middle between the insertion of the inner and outer surface of the tendon, using a precise preparation. All measurements were made after preconditioning before the mechanical testing at the initial load of 10 N. The method of processing caused no significant differences in tendon dimensions, except for a slight increase in the tendon area (Table 1). We measured the length 3 times with a micrometer and recorded in the average value. Variations in repeated measurements of length at the same location were less than 1.3%. The cross-sectional area of the entire ligament was measured with an area micrometer. This consisted of a torque-measuring thimble, a plug with a rectangular blade and a defined block system that surrounded the ligament with a blade pressure of 0.12



Fixation device for the patellar tendon.

MPa, which was applied for 2 minutes (Table 2) (Butler et al. 1985).

Mechanical testing

Mechanical testing was done after rehydration or thawing (group I) in Ringer's solution for 24 hours at 22 °C. The patella and tibia were fixed to a special frame (Figure).

Alignment was achieved by transitory angle plates at the frame edges. Temporary Halo screws prevented slipping of the specimens during drilling. The loading screws were drilled during controlled fixation in an outer polyethylene mold to ensure drill precision. The patella was also fixed with two crossed k-wires, connected by polymethylmethacrylate (PMMA), but not including the ligament, to avoid changes in the local temperature in the ligament. The direction of load was along the longitudinal axis of the ligament. The ligament was stretched in 10 preconditioning cycles to a maximal load of 100 N for 5 sec, until the hysteresis stabilized, to minimize creep. After preconditioning, the resulting extension at 10 N was defined as the initial ligament length, LO (mm). Mechanical testing was done with a material-testing machine (model 748; Wolpert 1 TTZ, Germany) at constant temperature (22 °C). Deformation and change in the tendon length were measured by using the frame displacement of the machine. The tendons were permanently wrapped with saline-moist towels during the whole experiment. Data were recorded with an integrated electromechanical system. The deformation rate was 15 mm/min (0.25 mm/s). The mode of failure in each specimen was noted. On the basis of the load displacement curves, four properties were studied: failure stress (maximum force divided by initial tendon cross-section), failure strain (elongation divided by initial length), normalized stiffness (slope of the linear portion of the force-deformation curve) and energy at failure (area under the force elongation curve to maximum force) (Butler et al. 1985).

Statistics

We performed an ANOVA analysis of variance (one-way, multifactorial) between and within the groups, based on normally distributed data. When this analysis showed a difference at a 5% level, then we used the paired test with Scheffe's method to evaluate group differences. The homogeneity of variances was controlled by the Levene test. Groups I and II were compared with the unpaired Student t-test. The statistical analysis was done, using the SPSS software package (version 6.1.3, SPSS Inc., Chicago, IL).

Results

Modes of failure

We found no differences in type of failure between the two groups. In group I (deep-frozen), we had 8 cases of midsubstance ligament rupture. In 1 case, periosteal failure occurred near the insertion of the ligament in the patella and in another, a tibial avulsion fracture. In the freeze-drying control group, 9 cases had ligament failure and 1 a tibial avulsion fracture. In group II (lipid extraction, disinfection, freeze-drying), we had 8 cases of midsubstance ligament rupture, 1 of periosteal failure near the patella insertion and 1 patellar avulsion fracture. In the freeze-drying control group, we found 8 cases of midsubstance ligament rupture and 2 of patellar avulsion fracture.

Table 2. Mechanical properties of patellar tendons. Values are given as mean (SD)

	Group I Deep-frozen	Control Freeze-drying	P-value	Group II Lipid extraction, disinfection, freeze-drying	Control Freeze-drying	P-value
Failure stress (MPa)	53 (14)	26 (15)	0.04	49 (13)	28 (5)	0.08
Failure strain (%)	21 (3)	16 (11)	0.7	18 (3)	18 (8)	0.9
Normalized stiffness (MPa)	223 (84)	151 (85)	0.5	271 (45)	176 (59)	0.2
Energy to failure (Nm)	8 (3)	6 (4)	0.9	7 (3)	5 (3)	0.8

Mechanical properties (Table 2)

Only failure stress was found to differ significantly between and within the groups (p = 0.04), but failure strain (p = 0.6), normalized stiffness (p = 0.2) and energy to failure (p = 0.9) were similar. The additional paired t-test showed a significant improvement in failure stress in the comparison of the deep- frozen tendons (group I) to freeze-dried controls, but in the lipid-extracted, disinfected and freeze-dried tendons (group II) the difference was not significant. Using the unpaired test, comparison of the deep-frozen tendons (group I) and the lipid-extracted, disinfected and freeze-dried tendons (group II) likewise showed no significant difference (p = 0.8).

Discussion

Our study shows that the initial mechanical properties of the patellar tendons, using extended processing, are as good as those for deep frozen tendons. Previous studies concluded that, in a comparison with fresh specimens, freezing had little, if any, effect on the mechanical properties (Woo et al. 1986, Silver et al. 2000). In this respect, we can therefore recommend this extended processing method for handling soft tissue.

We chose an in vitro analysis in sheep instead of a human cadaver study because we wished to compare the data with findings after transplantation. In other studies, the sheep model has also been used for anterior cruciate ligament transplantation and similar initial mechanical data were found (Jackson et al. 1988, Zimmerman et al. 1991). This model was regarded as comparable to human anatomic conditions. One limitation of our study is that we did not make local strain measurements of the ligament itself. Therefore, our findings do not reflect "real ligament properties" sufficiently (i.e., normalized stiffness, failure strain and energy) (Butler et al. 1985). Our data also include changes from bone deformation and inhomogeneity of the ligament structure. We accepted these effects because we were primarily interested in the mechanical and structural properties of the entire bone-ligament-bone unit used clinically (Jackson et al. 1990, Shino et al. 1990, Tohyama and Yasuda 1998). Tissue fixation and slippage problems, often noted in ligament testing, were avoided by a special bone screw fixation. Avulsion fractures occurred only rarely and could be explained by our slow strain rate of less than 1% per second. Therefore, the results are more relevant in slow tendon loading situations than in high speed trauma situations in sports medicine that need strain rates of 100% per second (Noyes et al. 1974).

The effects of freeze-drying on the initial mechanical properties of ligaments have been the subject of discussion. Theoretically, the collagen structure might be destroyed by the growth of ice crystals (Turner et al. 1988). However, Czitrom et al. (1985) and Jackson et al. (1990) recommended freeze-drying as the best method for processing bone and ligaments. Thomas and Gresham (1963) found no significant differences in mechanical properties between the deep-frozen and freeze-dried fascia lata. Turner et al. (1988) reported no change in mechanical properties, apart from an increase in ligament elongation. His results may have been affected by the use of distilled water instead of 0.9% NaCl for rehydration. Bechthold et al. (1994) described significant greater failure stress and failure strain data in human frozen patellar tendons than in freeze-dried tendons. These results also support our finding that the frozen tendons are better by a factor of two. The surface disinfectant iodoacetic acid was first recommended only for bone processing by Urist et al. (1975). The effect on freeze-dried ligaments has not been analyzed before. In our study, normalized stiffness and energy improved slightly. These findings are difficult to explain and the influence of a collagen-linking effect of iodoacetic acid may need further micromorphological investigation. This processing method may provide an additional security against infectious loading of allografts, because iodoacetic acid proved to be effective against bacteria and virus (Lopez et al. 1990, Von Versen et al. 1990). However, the qualities of penetration into bone mineral have not yet been adequately clarified, but in ligaments this is easier to achieve. Von Versen et al. (1990) found that application of a vacuum (20 kPa) could improve this shortcoming. The processing method described here is not entirely consistent with current tissue bank procedures. Some banks use freeze-drying in combination with a preliminary rinse in ethanol or solvent agents. This approach also provides good initial mechanical properties, decontamination and preservation of osteoinductive properties (Delloye et al. 1991, Maeda et al. 1993, Zimmerman et al. 1994).

No competing interests declared.

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