The influence of freezing on the tensile strength of tendon grafts: a biomechanical study

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Our goal was to investigate the influence of freezing on the tensile strength of fresh frozen tendon grafts. The biomechanical characteristics of tendons that are less commonly used in knee surgery (Tibialis Anterior, Tibialis Posterior, Peroneus Longus and medial and lateral half of Achilles tendons) were compared to those of a semitendinosus and gracilis graft harvested from the same 10 multi-organ donors.
All right side tendons constituted the study group and were frozen at –80°C and thawed at room temperature 5 times. All left side tendons were frozen at –80°C and thawed at room temperature once. There were 59 tendons in the control group and 56 in the study group. The looped grafts were clamped at one1 side using a custom-made freeze clamp and loaded until failure on an Instron 4505 testing machine.

The average ultimate failure load in the control and study group was not significantly different between the control and the study group ($p>0.05$). The failure load of the medial tendon Achilles was the lowest in both study and control group ($p<0.001$). There was no statistical significant difference in maximum stress, maximum displacement, maximum strain and stiffness between the control and study group ($p>0.05$).

From our study, we conclude that freezing tendons at –80°C and thaw several times does not influence the maximum load, maximum stress, maximum displacement, maximum strain and stiffness. The medial half of the Achilles tendon is statistically the weakest tendon ($p<0.001$).
Tendon grafts can be frozen at –80°C and thawed at room temperature several times without altering their biomechanical properties.

Key words: Anterior cruciate ligament – tendon graft – biomechanical properties – fresh frozen – cadaveric – tensile study

INTRODUCTION

It has been our experience that different tissue banks employ different protocols in the preparation of tendon grafts. Some tissue banks harvest from multi-organ donors and subsequently clean, prepare and pack the grafts for later clinical use. Using this method grafts are frozen only once, however the procedure of harvesting is longer and the risk of contamination is higher. Other tissue banks harvest grafts and immediately freeze them to -80ºC. Further preparation of the grafts takes place after all serological and bacteriological tests are negative. This procedure requires freezing and thawing at room temperature twice. The question arises as to whether repeated cycles of freezing and thawing have a deleterious effect on the biomechanical properties of tendon grafts.
In clinical practice allograft material may occasionally be thawed but not unpacked or used.

Is it then appropriate to return and refreeze this material for future use without an unacceptable deterioration of the biomechanical properties of the allogeneic material? Repeated freezing and thawing also occurs in 'in vitro' biomechanical testing.
Evidence of tissue damage from extracellular ice crystals, formed during freezing, has been shown in smooth muscle studies to be due to the formation of ice between muscle bundles trapping and cutting the fibers *(11).* The formation of ice crystals is influenced by the rate of cooling. Small tissue samples have a cooling rate of 2-3°C/min when placed at –80°C, while massive allografts have a slow rate of cooling (1°/min). This can give more ice formation and crushing of the fibers *(10,28).*
The purpose of this study is to determine whether repeated cycles of freezing and thawing results in any measurable deterioration in the biomechanical properties of the allogeneic tendon. We studied this effect in semitendinosus and gracilis tendons, generally used for grafting, and compared the effect of repeated cycles of freezing and thawing on other potential graft tendons.
To our knowledge, there is no data in literature on the influence of multiple freeze-thaw cycles on the biomechanical properties of grafts. The studies from Viidik *(38)* and Woo *(42)* have shown that one freeze-thaw cycle doesn't change the biomechanical properties of rabbit ligaments in comparison with fresh tendons.

METHODS

Tissue procurement and graft preparation

Semitendinosus and gracilis tendons (STG), tibialis anterior tendon (TA), tibialis posterior tendon (TP), peroneus longus (PL) and the Achilles tendon (divided in a medial (ATM) and lateral half (ATL) were harvested) from both legs of 10 multi-organ donors (range 47-70y; median 59y) (9 male 1 female)

The tendons from the right hand side formed the test group and were frozen at –80°C and thawed in saline of 37°C for 5 times. The left hand side tendons served as the control group and were frozen and thawed at room temperature in saline only once. From the control group 1 STG and from the study group 4 STG were damaged during prelevation, leaving 115 grafts for testing.

After removal of the bone block, the Achilles tendon was divided in a medial and lateral halve. All specimens were looped and the free ends were braided side-to-side under tension on a workstation (Acufex, Smith&Nephew, Memphis, TE, USA) with #1 Vicryl (Ethicon, Johnson & Johnson, Somerville, NJ, USA) using a whipstitch. Braiding was done as in clinical use and was standardized for all specimens. The diameter of the braided grafts was measured by using a graft sizer. Graft sizers with incremental diameters of 0.5mm were used. The folded end was placed over a 6mm metal rod, while the free ends were clamped in a custom-made freeze clamp. (fig. 1). In this way, the tendons were able to slide over the bar, which allowed equilibration of the tension between the 2 strands *(13)*. Care was taken to have the folded end of the graft 6 cm outside the clamp. In this way, the clinically relevant mid-portion of the graft was tested. Dry ice (-80°C) was inserted in the receptacles of the tendon clamp. The tendon was allowed to freeze until freezing was observed just outside (1-2mm) the clamp *(26)* (fig. 2).

All specimens were mounted on an testing machine (Model 4505, Instron Corp., Canton, MA, U.S.A.) with a 5 kN load cell. After a preload of 5 N *(9),* the grafts were pulled to failure at a rate of 1 mm/s, similar to strain rates reported in other studies *(17,26,41*).

Data analysis

The maximum load and displacement at maximum load were recorded. Stiffness was measured in the linear part of the curve. The maximal stress was calculated by dividing the maximal load by the calculated area surface of the graft. The maximal elongation divided by the free length outside the clamp gave us the maximal strain.

Statistical analysis

Data were analyzed using a Wilcoxon paired *t*-test for comparing the results of the study group with these of the control group (significance set at $p<0.05$). For comparison between the different tendons, a Wilcoxon unpaired *t*-test was used. To reduce the number of false-positive results, a Bonferoni adjustment of the p-value was used ($p<0.005$).

An initial power analysis. An initial power analysis with $\beta= 0.1$ and $\alpha=0.05$ showed that, in order to find a difference of 1%, both groups should contain more than 20000 specimens. For a 10% difference (with the same α and β) both groups should contain more than 200 specimens.

RESULTS

Clinical features of control and study group

The mean length of the tendons before folding, respectively for control and study group, was for STG 25.2 +/- 2.4 and 23.6 +/- 4.35 cm; for TA 30.5 +/- 1.35 and 30.1 +/- 2.81 cm; for TP 28.7 +/- 3.59 and 26.2 +/- 3.19 cm; for PL 31.2 +/- 1.14 and 29.9 +/- 2.81 cm; for ATM 25.4 +/- 2.37 and 24.6+/- 2.12 cm; for ATL 25.3 +/- 1.77 and 24.6 +/- 2.17 cm. The mean diameter of the tendons from control and study group was 8.51+/-0.8 en 8.42+/-0.7 mm with p=0.49 (Wilcoxon paired *t*-test).

There was no significant difference between the tested or control tendons in any of the anatomical sites studied in terms of length or cross-sectional area.

Maximum load to failure

The results of the maximal load to failure are presented in table I. No overall statistically significant difference was identified between the test and control. The maximal load to failure of ATM was statistically significantly weaker in both the test and control groups (table I).

The maximal stress is presented in figure 3. There was no statistically significant difference ($p>0.05$) in maximal stress between the study and control group (table II) in any of the tendon groups studied.

In the study group, the maximal stress of ATM and ATL was significantly lower ($p<0.001$) than these of TA and TP.

Maximal elongation

The maximal elongation is presented in figure 4. There was no statistically significant difference demonstrated between the study or control tendons ($p>0.05$) in maximal displacement (table II).

In the control group, the maximal elongation of ATM was significantly less ($p<0.001$) than STG and ATL. In the study group, the maximal elongation of ATM was significantly less ($p<0.001$) than these of STG, TA and PL.

Maximal strain

There was also no statistically significant difference ($p>0.05$) between the control and study group (table II).

In the control group, the maximal strain of ATM was significantly less ($p<0.001$) than ATL. In the study group, the maximal strain of ATM was significantly less ($p<0.001$) than STG, TA and PL.

Stiffness

The control and study group were not statistically significantly different ($p>0.05$) (table II). In the control group, TA were significantly stiffer than STG and ATM ($p<0.001$). TP were also significantly stiffer than ATM ($p<0.001$). In the study group, TA were significantly stiffer than ATM and ATL ($p<0.001$).

DISCUSSION

Methodological Issues

Gibbons *et al* have *(9)* demonstrated that right and left tendons from the same donor exhibit the same tensile properties. Because the clinical features of the left and right tendons were equal, we have assumed that the initial biomechanical properties of left and right tendons before treatment were the same.

Initial power analysis showed that, in order to find a 1% difference, each group should contain more than 20000 specimens. For a 10% difference, there should be more than 200 specimens in each group. As the availability of multi organ donors is limited, we could only use tendons from 10 donors for our study.

The difficulty in testing tensile properties of tendons without bone block, is gripping the tendon without slippage and without damaging the tendon fibers. We used a custom-made freeze clamp, as recommended in the literature *(8,12,13,20,26,29,30)*. Any tearing at the insertion into the clamp may result in lower measurements for strength and stiffness. We occasionally observed slippage at the site of insertion into the clamp. Therefore, the results for maximal load, maximal stress and stiffness may have been slightly underestimated.

We used a strain rate of 1mm/sec, similar as used in other studies *(17,26,41).* This corresponds to a strain rate of approximately 5%/sec. Some authors have recommended a strain rate of 100%/sec, since this rate is thought to produce soft tissue disruption before bony avulsion occurs *(22,40).* As we did not use bone-tendon-bone specimen, the effect of stain rate on failure mode is not a factor in this study.

For this study, we had no area micrometer available as described by Walker *(39)* and Ellis *(7).* Because we used a graft sizer on all grafts, we can compare the results of maximum stress between the different grafts. Areas measured by one method tend to differ from those measured by another by a constant factor K *(7).* This needs to be kept in mind when the results are compared with the literature.

From a tissue banking point of view, it is easier to harvest the tendons during the multi organ donor surgery and clean the tissues in a second stage after the period of quarantine.

To date, the most frequently used tendons for ACL-reconstruction include bone-patellar tendon-bone block (*1-3,14,18,21,25,27,33,34,37,43),* quadriceps tendon, hamstrings tendons and Achilles tendon *(15,19,25,27,33).* There are fewer reports on the use of tibialis anterior, tibialis posterior or peroneal tendons *(4,13,26).* In this study, the biomechanical properties of STG, TA, TP, PL and Achilles tendon (ATM, ATL) were determined and can be compared with the literature.

Woo *et al.(41)* demonstrated that the tensile strength of native ACL decreases with aging. The average age of the grafts was 59 years (range 47-70y). In clinical use, specimens under 40 years are usually required to provide adequate biomechanical strength for ligamentous reconstruction. Because our tensile tests still showed high values, we therefore agree with Pearsall *et al (26*) that older specimen still have sufficient biomechanical strength to be considered as potential candidates for allograft donation, thereby increasing the donor pool of these grafts.

Increasing the cross-sectional area of the graft gives higher values for maximal load *I6).* For ACL reconstruction, increasing the cross-sectional area is limited by the space available in the intercondylar notch. In order to be comparable with clinical use; we therefore decided to split the Achilles tendon in a medial and lateral half and thinning out the grafts until their diameter was less than 10 mm.

Influence of freezing

To our knowledge, there is no data in literature on the influence of multiple freeze-thaw cycles on the biomechanical properties of grafts. The studies from Viidik *(38)* and Woo *(42)* have shown that one freeze-thaw cycle doesn't change the biomechanical properties of rabbit ligaments in comparison with fresh tendons. In our study, we found no statistically significant difference between the study and control group. We therefore conclude that multiple freezing does not influence the biomechanical properties of the allografts.

Suitability of the grafts for ligament reconstruction

To be suitable for ligamentous reconstruction, tendon allografts must have biomechanical properties equal or better than native ACL. We have considered only the initial mechanical properties of the allografts. After reconstruction, remodeling of collagenous tissue and revascularization occurs. Studies with animal models have demonstrated considerable weakening of biological grafts, compared with their initial strength *(1,5,16).* After ACL reconstruction, tibial fixation fails at a much lower force than the ligament *(32)* These issues are not considered in this study.

In literature, the maximal load of native ACL varies between 633 and 1725N, with reported stiffness ranging from 129 to 220 N/mm *(17,22,23,31,36,41).*

Studies testing bone-patellar tendon - bone grafts found values for maximal load from 1784 to 2977N and for stiffness from 210 to 519 N/mm *(3,6,22-24).*

The average maximum load of the STG grafts in our study was 3120N. In literature, maximum loads from 1216 N up to 4590 N are described *(8,12,13,20,22-24).* Tis *et al (35)* found lower values of maximal load (532N), but these tensile test were performed without freeze-clamps. Hamner *et al (12)* had the highest values of maximal load (4590N) , when the 4 strands were equally tensioned by the use of weights. They also reported a stiffness of 776N, while in other studies the reported stiffness of STG grafts range from 213 to 418 N/mm *(8,13,24).* Only Millet *et al (20)* found a stiffness of 1553 N/mm. In our study, the STG grafts were manually tensioned. This can explain the slightly lower values.

The maximal load was the highest in the TA tendons with an average of 3980N. Some grafts even had maximal loads exceeding 5 kN. The average stiffness was 424 N/mm. These values are comparable with the literature, with reported maximal loads from 3421 to 4122 N and stiffness from 344-460 N/mm *(13,26).*

For TP grafts, our results for maximal load (3500N) and stiffness (392 N/mm) match those reported in literature with values of maximal load and stiffness of 3391 to 3594 N and 301 to 379 N/mm respectively *(13,26).*

The average maximal load and stiffness of PL were respectively 3420 N and 352 N/mm. This is comparable to the literature (2483N and 243N/mm) *(26).*

In literature, many reports exist on the use of Achilles tendon allografts for ligamentous reconstruction with good clinical outcome *(15,19,25,27,33).* We could not find reports on the biomechanical strength of fresh frozen Achilles tendon allografts. In our study, the lowest maximum loads were in the ATM group, where grafts failed at 560 N (table 1). We found that the so valued Achilles tendon is, in our tests the weakest tendon looking at all evaluated parameters when compared to TA, TP, STG and PL tendons.

CONCLUSIONS

Several conclusions can be made:
1. Repeated freeing and thawing of allograft does not influence biomechanical characteristics of allograft tendons.
2. The tibialis anterior, tibialis posterior, peroneus longus and hamstrings tendons have strong biomechanical properties and are therefore suitable for ligamentous reconstruction.
3. The biomechanical properties of the medial and lateral half of the Achilles tendon are less favourable in comparison with the results of the other tendons.

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LEGENDS

Figure 1: Freezing clamp
Figure 2: Test set-up
Figure 3: Comparison of maximum stress between the different tendons in control and study
Figure 4: Comparison of maximum elongation between the different tendons in control and study group.
Figure 5: Comparison of maximum strain between the different tendons in control and study group.
Figure 6: Comparison of stiffness between the different tendons in control and study group.

Table I: Maximum load to failure
Wilcoxon paired *t*-test ($p<0.05$)

Table II: Comparison of maximum stress, maximum displacement, maximum strain and stiffness . Wilcoxon paired *t*-test ($p<0.05$)

TABLES

Table I

	Control average	Control range	Study average	Study range	Statistic* difference
STG	3120 N	2370 N - 3870 N	3280 N	2440 N - 4040 N	p>0.2
TA	3980 N	2910 N - 5080 N	4300 N	3000 N - 5110 N	***p<0.05***
TP	3500 N	2820 N - 4520 N	3500 N	1780 N - 4330 N	p>0.2
PL	3420 N	2410 N - 4230 N	3350 N	2700 N - 4130 N	p>0.2
ATM	1690 N	560 N - 2800 N	1900 N	1000 N - 3120 N	p>0.2
ATL	2960 N	2330 N - 3990 N	2290 N	610 N - 3140 N	***p<0.01***

Table II

	Max stress	Max displacement	Max Strain	Stiffness
STG	p>0.2	p>0.2	p>0.2	p>0.2
TA	p<0.1	p>0.2	p>0.2	p>0.2
TP	p>0.2	p>0.2	p>0.2	p>0.2
PL	p>0.2	p>0.2	p>0.2	p>0.2
ATM	p>0.2	p>0.2	p>0.2	p>0.2
ATL	p<0.1	p<0.2	p<0.2	p<0.2

Figures

Figure 1

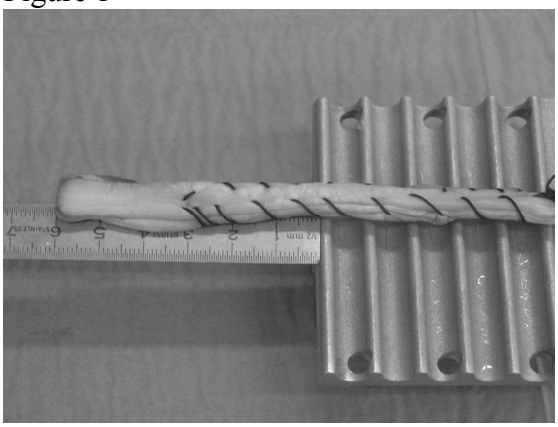

Figure 2

Figure 3

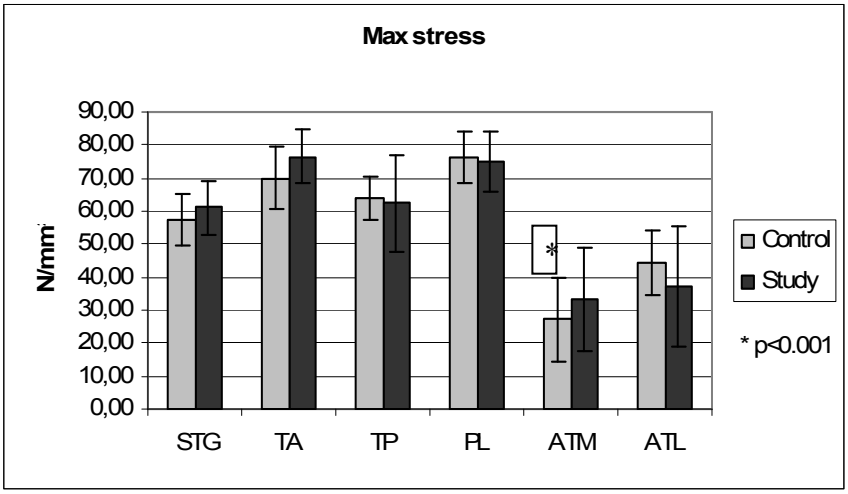

Figure 4

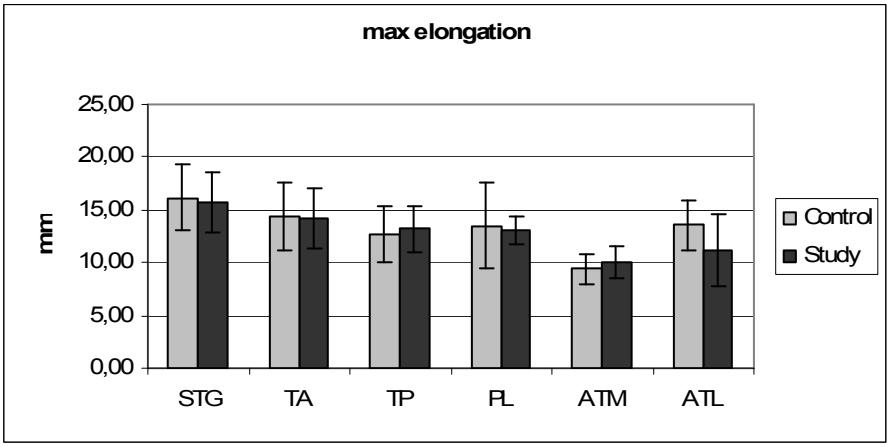

Figure 5

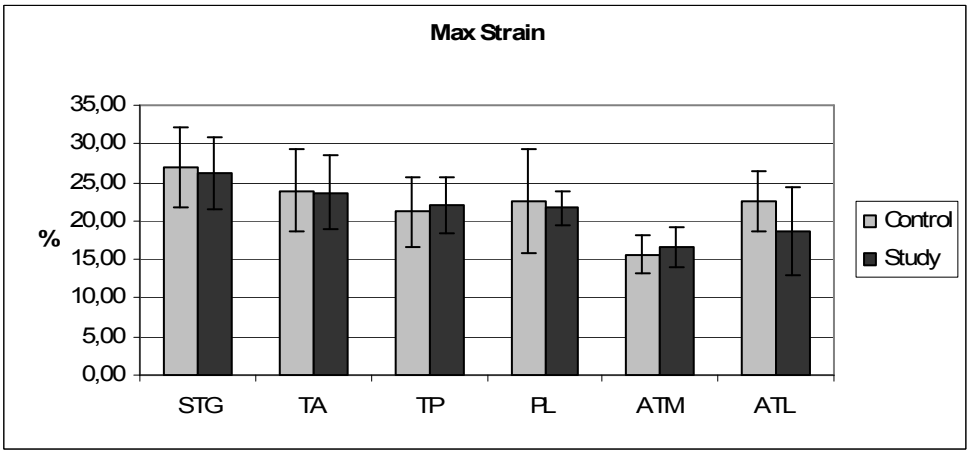

Figure 6