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Research Article

Effects of freezing, liquid nitrogen, and formaldehyde preservation on rat achilles tendon biomechanics

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ABSTRACT

Objective: This study aimed to compare the effects of different preservation methods, including −20°C freezing, −80°C freezing, liquid nitrogen, and 10% formaldehyde preservation, on the biomechanical properties of rat Achilles tendons.

Methods: This experimental biomechanical study included Achilles tendons obtained from 25 male Sprague–Dawley rats (age, 3 months; weight, 220-280 g). The rats were randomly allocated to five groups (n=5 each): fresh, −20°C freezing, −80°C freezing, liquid nitrogen preservation, and 10% formaldehyde preservation. Specimens in the preservation groups were stored for 1 week before biomechanical testing. Following preconditioning, the tendons were subjected to axial tensile loading at 3 mm/min until failure. Cross-sectional area, maximum elongation, maximum strain, maximum stress, maximum load, and stiffness were evaluated.

Results: Initial tendon length did not differ among the 5 groups ($P = .92$). Maximum load was comparable between the fresh (48.78 ± 2.35 N), −20°C (50.28 ± 2.57 N), and −80°C (48.95 ± 3.21 N) groups, whereas it was significantly lower in the liquid nitrogen (34.21 ± 2.98 N) and 10% formaldehyde (23.55 ± 2.39 N) groups ($P < .05$). Stiffness was significantly higher in the −20°C (32.99 ± 2.37 N/mm) and −80°C (30.74 ± 1.63 N/mm) groups than in the fresh (25.80 ± 1.11 N/mm), liquid nitrogen (23.21 ± 1.27 N/mm), and 10% formaldehyde (19.66 ± 1.36 N/mm) groups ($P < .05$). The 10% formaldehyde group also showed significantly lower maximum elongation, maximum strain, and maximum stress than the cryopreservation groups ($P < .05$).

Conclusion: Preservation at −20°C and −80°C maintained maximum tendon load at levels comparable to fresh tendons but resulted in increased stiffness. Liquid nitrogen and 10% formaldehyde preservation were associated with lower maximum load, with the most pronounced alterations observed after formaldehyde preservation. These findings indicate that the choice of preservation method should be considered when interpreting biomechanical tendon studies, as different preservation methods may affect individual biomechanical parameters differently.

Introduction

Tendons are organs that connect muscle to bone, and in some cases, they connect one muscle belly to another as intermediate tendons.[1] Entheses are biomechanically complex regions where tendons or ligaments attach to bones.[2] Native tendons primarily experience tensile stimulation as their dominant mechanical loading mode, with the force transduction mechanism mediated by muscular contraction-induced tension.[3] The extracellular matrix of healthy tendons consists predominantly of collagen, which accounts for 60%-85% of its dry mass.[4] Approximately 95% of the collagenous proteins in this matrix are type I collagen.[5] Cycle strains may also affect the composition of tendons.[6]

Over 15 million people worldwide sustain tendon injuries each year.[7] Conservative treatment or surgical repair alone does not always result in full recovery.[8] Tendon transplantation is now a common treatment for tendon injury and can restore joint stability and function, thereby helping to maintain patients' quality of life.[9] The sources of tendons in tendon transplantations are categorized as autografts or allografts. Autografts may be harvested from various sites, such as the hamstring tendons and the iliotibial band. Allogeneic tendon grafts are widely used in clinical settings because of their ability to avoid secondary injuries, the efficiency of the surgical procedure, and effective repair outcomes.[10] Tendons from different sources may exhibit distinct biomechanical properties.[11] Allogeneic tendon transplantation and tendon-related experimental research both require effective preservation techniques to maintain structural and functional integrity. Different preservation techniques may adversely affect these properties.[12] Moreover, the viability of allograft tendon grafts gradually decreases after harvesting, thus highlighting the critical importance of long-term tendon preservation.

Many researchers have reported the effects of repeated freeze–thaw cycles and preservation duration on the mechanical properties of tendons, with freeze–thaw temperatures including −80°C, −40°C, and −20.[13,14] The effects of irradiation on tendons' mechanical properties have also been reported.[15] However, few studies have addressed the effects of liquid nitrogen and formaldehyde on the biomechanical properties of tendons. Hohmann's study investigated

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the load to failure and Young's modulus of elasticity in fresh, fresh–frozen, Thiel-preserved, and formalin-embalmed long heads of biceps tendons, while Beger's study compared 7 preservation methods for tendons and muscles to investigate biomechanical properties and histological changes.[16,17] However, neither of these studies included different degrees of cryopreservation or the liquid nitrogen method. Accordingly, the present study was designed to compare differences in the biomechanical properties of tendons preserved by 10% formaldehyde, −20°C freezing, −80°C freezing, liquid nitrogen, and fresh tendons to identify the most suitable preservation method.

Material and methods

This study protocol was approved by the Medical Ethics Committee of Tianjin Hospital (Approval no.: 2025-Medical Ethics Review-200) on February 4, 2026. This study was conducted on December 1, 2025. Informed consent was not required because this was an animal study.

Twenty-five male Sprague–Dawley (SD) rats (weight: 220-280 g, age: 3 months) were obtained from the Tianjin Experimental Animal Center. The animals were randomly assigned, using a random number table, to 5 groups: fresh, −20°C, −80°C, liquid nitrogen, and 10% formaldehyde.

Tendon harvesting and preservation

Bilateral Achilles tendons (length: 10-12 mm) in the fresh group were harvested from euthanized rats, and biomechanical testing was performed within 1 hour, with normal saline applied by spraying during the process. Achilles tendons in the −20°C group were wrapped in double-layer gauze saturated with normal saline after harvest, placed in a plastic bag, and then stored in a refrigerator (4°C) for 20 minutes. They were then cooled to −20°C at a rate of 0.5-1.0°C/min and preserved for 1 week before testing. Achilles tendons in the −80°C group were wrapped in double-layer gauze saturated with normal saline after harvest, placed in a plastic bag, and then stored in a refrigerator (4°C) for 20 minutes. They were then cooled to −80°C at a rate of 0.5-1.0°C/min and preserved for 1 week before testing. Achilles tendons in the liquid nitrogen group were placed in a frozen pipe with cryoprotectant, immediately transferred to liquid nitrogen cryovials after harvest, and preserved for 1 week before testing. Achilles tendons in the 10% formaldehyde group were immediately placed in 10% formaldehyde after removal and preserved for 1 week before testing.

The Achilles tendons preserved at −20°C and −80°C were thawed for 6 hours at room temperature (21°C) and sprayed constantly with normal saline to keep them moist. The Achilles tendons preserved in liquid nitrogen were put in a thermostat water bath at 37°C for 1 to 2 minutes until thawed. After thawing, the tendons were immediately rinsed with normal saline to remove any residual protective agents from the surface before biomechanical testing. Tendons preserved in 10% formaldehyde were extracted and dried with gauze before testing.

HIGHLIGHTS

- Preservation at −20°C and −80°C maintained maximum tendon load at levels comparable to those of fresh tendons but resulted in increased stiffness.
- Liquid nitrogen and 10% formaldehyde preservation were associated with a lower maximum load, with the most pronounced alterations observed after formaldehyde preservation.
- These findings indicate that the choice of preservation method should be considered when interpreting biomechanical tendon studies, as different preservation methods may affect individual biomechanical parameters differently.

Biomechanical testing

An Endura TEC ELF 3200 (Endura TEC Systems Corporation, Minnetonka, MN, USA) was used for biomechanical testing. The tendons were placed on the machine along its loading axis, with approximately 5 mm between the 2 fixtures. The length and width of the proximal, mid, and distal transverse sections of each tendon were measured using a digital vernier caliper (accuracy: 0.1 mm) before testing. The values of these 3 transverse sections were averaged to calculate the tendon's transverse area.

To freeze the tendons to the fixtures to prevent sliding, both ends of the tendons were fixed with frozen fixtures, which were secured with 2 screws and cooled by a circulating cooling system. An axial tensile preload (0.5 N) was applied to straighten each tendon. An axial tensile load from 0 N to 8 N was then applied at 3 mm/min for 20 cycles to reduce the error from tissue creep. Although one study reported a preload of 50 N maintained for 2 minutes, that study used posterior tibial tendons harvested from human donors, which are considerably larger than the SD rat Achilles tendons used in the present study; hence, the preload was reduced accordingly.[18] After tensile loading, the tendons were straightened and the machine was zero adjusted. The distance between the 2 fixtures was then measured with a vernier caliper as the initial tendon length. Subsequently, tendons were loaded at 3 mm/min again until failure, defined as a rupture in the mid-region or as the load-displacement curve reaching its peak. The maximum load and maximum elongation (defined as the change in length from the initial length at failure) were recorded by the software; maximum strain and stress were calculated using the initial length and transverse area (maximum strain [%]=maximum elongation/initial length × 100, maximum stress=maximum load/transverse area). Tendon stiffness is represented by the slope of the linear part of the load–elongation curve (Figure 1).[19] All testing and measurements were conducted at 26°C and 65% humidity.

Statistical analysis

Statistical analysis was performed using SPSS 16.0 (SPSS, Chicago, IL, USA). Data on the mechanical properties for each group are presented as $\bar{x} \pm s$. A 1-way analysis of variance was performed on the experimental data, and Tukey's post hoc test was used for pairwise comparisons. Differences were considered statistically significant at $P < .05$.

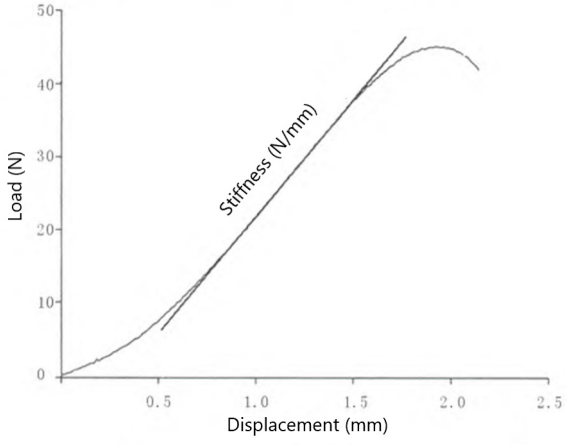

Figure 1. Stiffness calculated from the load–displacement curve. The slope of the curve is stiffness.

Results

Initial length and transverse area

The initial length of the tendons in all groups was approximately 5 mm, with no statistically significant difference ($F=0.22$, $P=.92$) (Table 1). The transverse area of the cryopreservation groups (−20°C, −80°C, and liquid nitrogen) was larger than that of the fresh group, and the differences were statistically significant ($P < .05$). The transverse area of the tendons in the 10% formaldehyde group was smaller than that in all other groups, and the differences were statistically significant ($P < .05$).

Maximum elongation, maximum strain, and maximum stress

Compared with the fresh group, the maximum elongation, maximum strain, and maximum stress of the tendons in the other 4 groups showed varying degrees of reduction, and the differences were statistically significant ($P < .05$). However, no statistically significant differences were observed among the −20°C, −80°C, and liquid nitrogen groups. The 10% formaldehyde group exhibited statistically significantly lower values than the cryopreservation groups (−20°C, −80°C, and liquid nitrogen) for all 3 parameters ($P < .05$) (Table 1).

Maximum loading

Compared with the fresh group, the maximum loadings of the −20°C group and the −80°C group increased, but the differences were not statistically significant. The liquid nitrogen and 10% formaldehyde groups showed statistically significantly lower maximum loading than the fresh group and the other groups ($P < .05$). No statistically significant differences were observed between the −20°C and the −80°C groups (Table 1).

Stiffness

The 10% formaldehyde group had the lowest stiffness among all groups and was statistically significantly lower than that of the other 4 groups ($P < .05$). The −20°C and −80°C groups had statistically significantly higher stiffness than the fresh, liquid nitrogen, and 10% formaldehyde groups ($P < .05$). However, there was no statistically significant difference between the −20°C and −80°C groups ($P > .05$), nor was there any statistically significant difference between the stiffness of the liquid nitrogen group and the fresh group (Table 1).

Discussion

This trial aimed to compare the effects of different preservation methods on Achilles tendons in SD rats to provide better guidelines for clinical and experimental researchers. Statistical analyses showed no significant differences in initial tendon length among the groups. Notably, the transverse areas of the tendons in the cryopreservation groups increased, indicating that the tendons preserved by these methods swelled to various degrees, primarily due to an increase in the diameters of the tendon fibers and the spaces between the fibers.[18] Other researchers have found that tendon swelling is related to crystal formation when the temperature decreases.[20,21]

Cryopreservation groups

Cryopreservation is widely used as a preservation technique for tendons. However, whether −20°C, −80°C, or liquid nitrogen is the best method remains a subject of debate. Several studies have found that the maximum stress of cryopreserving tendons is lower than that of fresh tendons.[20,22] Other trials have found that cryopreservation does not affect the biomechanical properties of tendons.[13,14] The results of the study are consistent with the latter reports. The maximum loading in the −20°C and −80°C groups increased slightly compared with the fresh group, but the differences were not significant, which is consistent with Lansdown et al.[23] Chen et al[21] found that reduced mechanical properties may result from crystal formation within the tendons caused by low temperatures. To explain why the stiffnesses of tendons in the −20°C and −80°C groups increased, which is a finding similar to that of Lansdown et al,[23] further research on tendon tissues may be necessary. Although Blaker et al[13] compared the biomechanical properties of kangaroo tendons preserved at −20°C and −80°C, they did not report tendon stiffness. The study addresses this disparity.

Liquid nitrogen group

Maximum elongation, maximum strain, maximum stress, and maximum loading of tendons were reduced in the liquid nitrogen group compared with the fresh group; however, no significant difference in stiffness was observed compared with the fresh group. The reduction in maximum elongation is consistent with Ohno et al.[24] This may be attributed to tissue damage caused by ice crystal formation within the tendons.[21] Interestingly, maximum strain and stress did not differ significantly between the liquid nitrogen group and the −20°C and −80°C groups, which is consistent with Oswald et al.[25] However, maximum load and stiffness were significantly lower than in the −20°C and −80°C groups. This may be related to both the low temperature and the differences in cooling protocols. The cooling velocity in the −20°C and −80°C groups was 0.5°C/min to 1°C/min, but the tendons in the liquid nitrogen group were immersed in liquid nitrogen immediately after removal from the rats. The cooling process of the liquid nitrogen group was intense and rapid. During this process, water in the tendons and cells froze into crystals, which may have caused more damage to the tendons.

Ten percent formaldehyde group

Compared with the fresh group, the transverse area and biomechanical properties of the tendons decreased in the 10% formaldehyde group. While these findings suggest that formaldehyde preservation may have caused tissue shrinkage or dehydration, this should be interpreted only as a possible explanation, because the study did not directly assess pre- and post-preservation dimensions, histology, or hydration status. However, Malik found that tendons preserved in 10% formaldehyde displayed peak loads and failure modes similar to those of fresh tendons, and Hohmann found that formalin-preserved

Table 1. The comparisons of initial length, transverse area, maximum elongation, and biomechanical properties of tendons among the groups ($\bar{x} \pm s$, n=5)

Groups	Initial length (mm)	Transverse area (mm²)	Maximum elongation (mm)	Maximum strain (%)	Maximum stress (N/mm²)	Maximum loading (N)	Stiffness (N/mm)
Fresh	5.06 ± 0.24	1.52 ± 0.06*	2.40 ± 0.27*	47.45 ± 4.29*	32.09 ± 1.76*	48.78 ± 2.35	25.80 ± 1.11
−20°C	5.15 ± 0.27	1.78 ± 0.22	1.85 ± 0.15	35.80 ± 8.00	28.12 ± 0.83	50.28 ± 2.57	32.99 ± 2.37
−80°C	5.19 ± 0.42	1.83 ± 0.11	1.74 ± 0.25	33.64 ± 4.00	26.64 ± 1.39	48.95 ± 3.21	30.74 ± 1.63
Liquid nitrogen	5.03 ± 0.18	1.77 ± 0.20	1.54 ± 0.13	30.68 ± 2.88	20.17 ± 0.90	34.21 ± 2.98*	23.21 ± 1.27
10% Formaldehyde	5.24 ± 0.17	1.35 ± 0.05*	1.12 ± 0.19*	21.39 ± 2.28*	17.24 ± 1.29*	23.55 ± 2.39*	19.66 ± 1.36*

The "*" indicates statistically significant pairwise comparisons described in the Results section ($P < .05$).

tendons and freshly frozen tendons had a similar load to failure.[16,26]. Therefore, formalin-preserved tendons may be useful in certain biomechanical settings, depending on the preservation protocol and outcome being tested. These findings differ from the results and may be attributable to differences in preservation duration and experimental protocols. Additional research on the biomechanics of tendons preserved in 10% formaldehyde is needed to resolve this controversy.

Limitations

This study has several limitations. First, as the use of SD rat tendons cannot fully replicate the tendons of other species or humans, the experimental results cannot be directly equated with those from clinical studies.[27] Second, there were differences in outcomes between this study and previous research. Histological and microscopic evaluations may help to explain these differences. Third, no sample size calculation was performed, and the small sample size may be the reason for the absence of statistically significant differences. Fourth, the preload protocol may have affected the biomechanical results because no supporting reference was available. Finally, the evaluation was conducted at a single time point; the long-term performance of the preserved tendons remains to be investigated.

In conclusion, tendons preserved at −20°C and −80°C may be more suitable for biomechanical studies because their biomechanical profiles are closer to those of fresh tendons than those of tendons preserved in liquid nitrogen or 10% formaldehyde. However, further histological and immunological studies are required to provide more evidence for allogeneic tendon transfer. In addition, for both biomechanical research and clinical tendon transplantation, further studies should examine the effects of preservation duration and animal model selection on tendon biomechanical properties and other biomedical parameters.

Data Availability Statement: The data that support the findings of this study are available on request from the corresponding author.

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