Annals of Anatomy 221 (2019) 186–191

Contents lists available at ScienceDirect

Annals of Anatomy

journal homepage: www.elsevier.com/locate/aanat

RESEARCH ARTICLE

The mechanical properties of fresh versus fresh/frozen and preserved (Thiel and Formalin) long head of biceps tendons: A cadaveric investigation

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ARTICLE INFO

Article history:
Received 14 February 2018
Received in revised form 9 April 2018
Accepted 2 May 2018

Keywords:
Biceps tendon
Thiel preservation
Formalin preservation
Fresh-frozen
Load to failure
Stiffness
Young's module

ABSTRACT

Human cadaveric specimens commonly serve as mechanical models and as biological tissue donors in basic biomechanical research. Although these models are used to explain both *in vitro* and *in vivo* behavior, the question still remains whether the specimens employed reflect the normal *in vivo* situation. The mechanical properties of fresh-frozen or preserved cadavers may differ, and whether they can be used to reliably investigate pathology could be debated. The purpose of this study was to therefore examine the mechanical properties of cadaveric long biceps tendons, comparing fresh (n = 7) with fresh-frozen (n = 8), formalin embalmed (n = 15), and Thiel-preserved (n = 6) specimens using a Universal Testing Machine. The modulus of elasticity and the ultimate tensile strength to failure was recorded. Tensile failure occurred at an average of 12 N/mm^2 in the fresh group, increasing to 40.1 N/mm^2 in the fresh-frozen group, 50.3 N/mm^2 in the formalin group, and 52 N/mm^2 in the Thiel group. The modulus of elasticity/stiffness of the tendon increased from fresh (25.6 MPa), to fresh-frozen (55.3 MPa), to Thiel (82.5 MPa), with the stiffest being formalin (510.6 MPa). Thiel-preserved and formalin-embalmed long head of biceps tendons and fresh-frozen tendons have a similar load to failure. Either the Thiel or formalin preserved tendon could therefore be considered as alternatives for load to failure studies. However, the Young's modulus of embalmed tendons were significantly stiffer than fresh or fresh frozen specimens, and these methods might be less suitable alternatives when viscoelastic properties are being investigated.

1. Introduction

Human cadavers are an important source of tissue specimens, often used to study their biomechanical properties (Maiden and Byard, 2016). Mechanical testing of human tissue is critical not only to establish the typical physical characteristics, but also to better understand their normal physiological behavior in response to mechanical loads (Griffin et al., 2016). For flexible elastic soft tissues, such as tendons and ligaments, the modulus of elasticity and the ultimate tensile strength best reflect their physiological behavior (Woo, 1982). In general, testing of fresh tissue is best suited to reproduce the natural properties in a reliable and repeatable fashion (Venkatasubramanian et al., 2006). However, acquiring fresh tissues for biomechanical studies is not always possible, and therefore, tissues preserved using methods such as cryopreservation (fresh-frozen) and embalming (Thiel or formalin) are most commonly used. Unfortunately, all of these preservation methods have been associated with inferior physical properties of tissues. For instance, it has been suggested that cryopreservation alters the stress–strain properties by damaging the extracellular matrix, with bulk redistribution of water by

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https://doi.org/10.1016/j.aanat.2018.05.002
0940-9602/

crystal ice growth (Venkatasubramanian et al., 2006; Chow and Zhang, 2011). Cartner et al. (2011) noted that specimen exposure time after thawing has a detrimental effect on tissue properties. In contrast, other researchers could not find any evidence that the physical and histological properties of fresh-frozen specimens were altered during cold storage (Panjabi et al., 1985; Bitar et al., 2010).

Working with human tissue has an inherent risk of disease transmission, and laboratories must be equipped to handle potentially infectious material (Greenwald et al. 2012; Lee et al., 2006). Although the risk of infection in allograft recipients is low, hepatitis B, hepatitis C, HIV, and group A streptococci are the most common organisms involved (Fishman et al., 2012). For this reason, embalming human cadavers became the method of choice as it significantly reduced the risk of disease transmission, but it may expose the researcher to chemical hazards and change the biomechanical properties substantially (Topp et al., 2012; Grizzle et al., 2012; Bell et al., 2010). Typically, human cadavers are embalmed with a combination of 96% ethanol and 2% formaldehyde perfused via the femoral artery and stored for at least one year (Topp et al., 2012). Formaldehyde overcomes the risk of infection but can alter the biomechanical properties, generally resulting in stiffer tissue (Hansen et al., 2009). As an alternative, the Thiel fixation technique has been developed to preserve the color, consistency, and transparency of human tissue (Thiel, 1992). The main solution used with this method consists of hot water, boric acid, ethylated glycol, ammonium nitrate, and potassium nitrate (Thiel, 1992). However, Thiel fixation has also been shown to affect the biomechanical properties of tissues. For example, studies demonstrated that this preservation method resulted in lower ultimate failure loads when compared to fresh-frozen tendons (Fessel et al., 2011; Liao et al., 2015). Conversely, Verstraete et al. (2015) recently reported an increase in stiffness for Thiel-embalmed Achilles' tendons.

Based on these conflicting results, it is not clear which preservation method would be the most appropriate for biomechanical studies. Therefore, the main goal of this study was to determine which preservation method best maintains the physical characteristics of freshly harvested tendons. The aim of this study was to investigate load to failure and Young's modulus of elasticity in fresh, fresh-frozen, Thiel-preserved, and formalin-embalmed long head of biceps tendons. We hypothesized that both Thiel and formalin preservation would increase the modulus of elasticity, as well as the ultimate failure load.

2. Materials and methods

The tendon of the long head of the biceps muscle was used in the study. Four groups comprising of fresh, fresh-frozen, formalin-embalmed, and Thiel-preserved tendons were included. The fresh tendons were sourced from patients who underwent routine arthroscopic rotator cuff repair or open shoulder replacement surgery. These specimens would normally be discarded, but verbal consent was obtained from all patients to allow their use for biomechanical testing. The fresh-frozen tendons were sourced from specimens that were used for a cadaveric shoulder course. Both formalin- and Thiel-preserved cadaveric tendons were obtained from the Department of Anatomy of the Ludwig Maximilian University of Munich, Germany.

2.1. Specimen preparation and storage

The fresh biceps tendons harvested during routine surgical procedures were stored in gauze soaked in Ringers lactate solution immediately following surgical resection. The excised tendons were transferred to the biomechanical laboratory and tested less than two hours after excision. As these resected tendons were removed for clinically related pain, and are often flattened and frayed due to the underlying pathology, the following inclusion criteria were applied: intact paratenon, smooth shiny tendon surface with no macroscopic fraying, and no obvious macroscopic flattening or splitting. A minimum length of 4 cm was required to be able to clamp the tendon into the holding frame.

The fresh-frozen samples were excised from cadavers used at a shoulder course held at the Department of XX. The specimens were cleaned of any remaining tissue, and wrapped in cotton gauze soaked in Ringers solution, inserted into a plastic bag, and frozen immediately to −30 °C. Fresh frozen specimens were stored for 10–14 days prior before being thawed at room temperature overnight. The same inclusion criteria were applied as for the fresh tendons.

The formalin-embalmed tendons were resected from cadavers that were used during the basic anatomy course of the department of Anatomy of the XX. The embalming fluid at the University of XX contained a 37% formaldehyde solution, and an average volume of 10 l was injected. For a 70 kg body this would result in a formalin concentration of approximately 5–6%. The bodies are kept in storage for 12 months after being embalmed and before distribution to the dissection halls. During the anatomy dissection, tendons, muscles, and their respective attachments and insertions were typically left intact. After harvesting, the specimens were wrapped with formalin-soaked gauze and transported to the biomechanical laboratory and tested the same day. Because of the preservation process, tendons typically lose their normal appearance, and visual differentiation between tendon and paratenon is not possible. Therefore, for this set of samples, the only inclusion criterion applied was an intact macroscopic appearance on visual inspection.

Thiel fixation was first described in 1992, as an alternative to formalin embalming (Thiel, 1992). This method is characterized by the preservation of color, consistency, and transparency of the tissue. Contrary to the usual formalin fixation, the main solution consists of hot water, boric acid, ethylated glycol, ammonium nitrate, and potassium nitrate, infused into the cadaver through a femoral artery catheter and a naso-gastric tube. Following infusion, the cadaver is stored in a container for a period of 12 months. The storage fluid in the container contains 1.5% formalin in addition to the above chemicals (Thiel, 1992). The specimens were sourced from the Department of Anatomy of the XX. The tendons were excised from cadavers, cleaned with saline, then transported in gauze soaked with preservation fluid and packed into sealed plastic bags. The bags were transported to the biomechanical laboratory and tested less than two hours later on the same day. As color, consistency, and transparency of the tissues are not disturbed in the preservation process, the same inclusion criteria as for the fresh and fresh-frozen groups were applied.

2.2. Measurements

The tendons were clamped into a holding frame (Fig. 1) that was secured with 6 screws (Fig. 2) and tightened with 5 Nm. The free tendon length between the clamping fixture was kept at 2 cm. Prior to measuring, the tendons were securely fastened into the testing machine and preloaded to 5 N. This technique ensured standardization, reducing the possibility of measurement error.

Length, width, and height of the tendons were then measured with a digital micrometer, having an accuracy of 0.1 mm. To further reduce variability between samples, all tendons were rectangular, and the measurements were taken at the smallest diameter, assuming this would be the weakest point where failure would most likely occur.

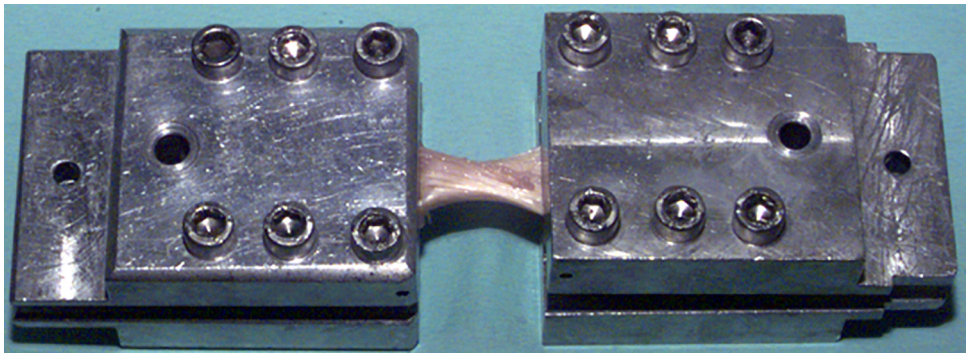

Fig 1. The tendons were clamped into a holding frame that was secured with 6 screws and tightened with 5 Nm.

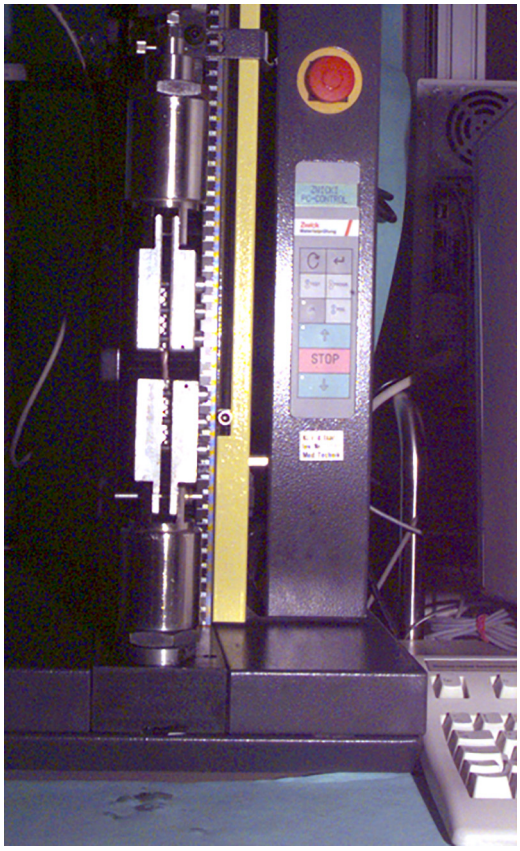

Fig. 2. The holding frames were securely fastened into the MTS machine and preloaded to 5 Newton.

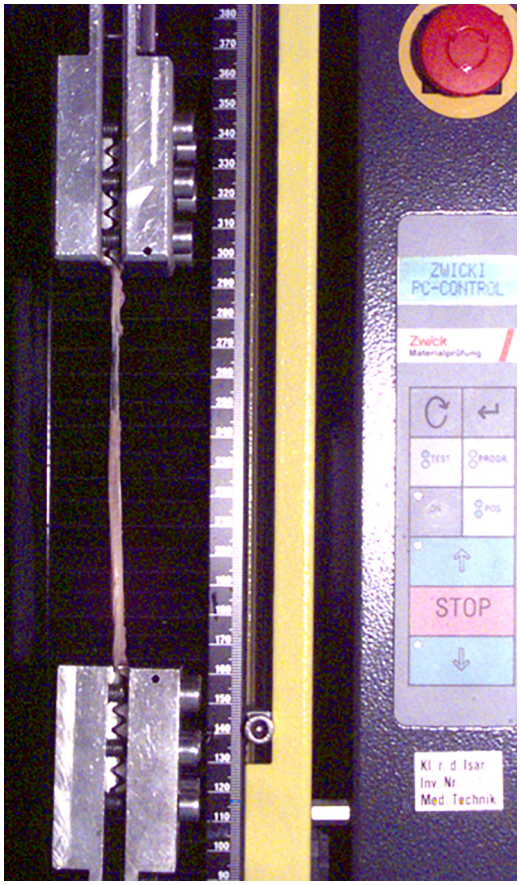

Fig. 3. Testing was performed at a speed of 10 mm/s until failure. Ultimate tensile strength and modulus of elasticity were calculated using automated software.

2.3. Testing protocol

Testing was performed at a constant room temperature of 22 °C with a humidity between 85–90%. Specimens were securely fastened into a servo-hydraulic testing machine (Zwick Universal Testing Machine, Zwick GmbH & Co.KG, Ulm, Germany) (Fig. 2), and following the preload of 5 N, each specimen was tested at a speed of 10 mm/s until failure (Fig. 3). This rate is representative of physiological activities and represents an approximate strain rate of 10% (Wren et al., 2001). Moistening during testing was not performed as the mean time from clamping to failure was less than 10 min. Ultimate tensile strength and modulus of elasticity were calculated using automated software.

2.4. Data analysis

The numeric parameters measured included the modulus of elasticity and the load to ultimate tensile failure. For analysis, the ultimate tensile load was corrected for cross section. An a priori sample size calculation was performed based on the recommendations by Christofolini (2000). Using a medium effect size, a significance level of 0.05, a power of 0.8, and a delta level of 20%, a minimum of 6 specimens per group were required. The Shapiro–Wilk test was used to determine whether sample data was normally distributed. Means and standard deviations were calculated for the dependent variables. One-way between group ANOVA with pairwise comparisons was used for analysis. All analyses were

Table 1
Sample size, mean age, and sample characteristics.

	Sample size n	Age Year	Width mm	Cross sectional diameter Sq mm
Fresh	7	62.4 + 7.2	8.7 + 2.5	4.0 + 1.3
Fresh-frozen	8	59.2 + 13.4	5.6 + 2.3	2.3 + 0.5
Thiel	10	81 + 12	5.1 + 0.5	2.2 + 0.9
Formalin	15	80 + 6.9	4.6 + 1.3	2.6 + 0.8

Table 2
Ultimate load to failure corrected for cross sectional diameter.

	Load to failure N/SQMM	SD	95% CI
Fresh	12	3.8	8.5–15.5
Fresh-frozen	40.1	12.3	23.1–57.1
Thiel	52	13.5	37.8–66.2
Formalin	50.3	15.5	41.7–58.9

Table 3
Young's modulus.

	Young's modulus (Pa)	SD	95% CI
Fresh	25.6	15.7	11.–40.1
Fresh-frozen	55.3	28.4	31.6–79
Thiel	82.5	38.3	42.3–122.7
Formalin	510.6	187.8	406.6–614.6

conducted using STATA SE (Version 12.0; StataCorp, College Station, Texas, USA) for Windows.

3. Results

Sample size, mean age, and the outcome measures are shown in Table 1. There were significant differences between the fresh specimens and the other three groups (fresh-frozen, Thiel, formalin) for mean width ($p < 0.001$) and cross sectional diameter ($p = 0.01$). There were no significant differences between the fresh-frozen, Thiel, and formalin groups. The fresh specimens were wider and demonstrated a larger cross-sectional diameter.

The ultimate load to failure, corrected for cross-sectional diameter, of the fresh tendons occurred at a mean of 12.0 ± 3.8 N/sqmm, for the fresh-frozen at 40.1 ± 12.3 N/sqmm, the Thiel specimens at 52.0 ± 13.5 N/sqmm, and the formalin specimens at 50.3 ± 15.5 N/sqmm (Table 2). One-way ANOVA revealed significant between-group differences ($p = 0.001$, $F = 100.7$). Comparisons between groups demonstrated significant differences between the Thiel and fresh groups ($p = 0.0005$, $F = 24.5$), fresh and fresh-frozen groups ($p = 0.005$, $F = 49.2$), and formalin and fresh groups ($p < 0.0001$, $F = 88.4$).

Young's modulus for the fresh tendons was calculated to be 25.6 ± 15.7 Pa for the fresh tendons, 55.3 ± 28.4 Pa for the fresh-frozen tendons, 82.5 ± 38.3 Pa for the Thiel-preserved tendons, and 510.6 ± 187.8 Pa for the formalin-embalmed tendons (Table 3), showing significant between-groups differences ($p = 0.002$, $F = 32.5$). Between-group comparisons demonstrated significant differences between the Thiel and formalin ($p < 0.0001$, $F = 27.8$), Thiel and fresh ($p = 0.016$, $F = 20$), formalin and fresh-frozen ($p < 0.0001$, $F = 38.6$), formalin and fresh ($p < 0.0001$, F=), and fresh-frozen and fresh groups ($p = 0.04$, $F = 29.9$).

4. Discussion

The results of this biomechanical study demonstrated that tendon specimens preserved by either Thiel or formalin fixation exhibit a comparable response in load to failure testing. Although the observed loads to failure were higher than in fresh-frozen specimens, significant differences were not observed. However, these loads were significantly higher when compared to fresh tendons. Surprisingly, we could not observe any differences between the preserved tendons and the fresh-frozen specimens. This would suggest that embalmed tendons could potentially be used as a substitute for fresh-frozen specimens when the main purpose is to investigate load to failure for biceps tendon, and possibly even general tendon testing.

In contrast, the findings for Young's modulus as a measure of elasticity has shown that embalmed tendons are significantly stiffer than fresh or fresh-frozen specimens, and are therefore not a suitable substitute when viscoelastic properties are the main outcome measure. Interestingly, there were also significant differences observed between fresh and fresh-frozen tendons, where fresh tendons had a lower Young's modulus.

Laboratory-based biomechanical research is largely dependent on the availability of human cadavers (Verstraete et al., 2015). Fresh specimens best represent *in vivo* biomechanical characteristics, but tissue decays rapidly post-mortem (Arnout et al., 2013). Fresh-frozen specimens are typically stored at −20 °C, and then thawed at room temperature prior to testing. Similar to fresh specimens, they can also only be used for a short period because putrefaction eventually occurs (Hocking and McIntyre, 2011). The main occupational risk is exposure to microbiological contaminants such as viruses and bacteria (Hayashi et al., 2016). In addition, the freezing process may change the structural properties of the tendons. Giannini et al. (2008) demonstrated a mean decrease in ultimate load by 18%, and showed no changes in Young's modulus in posterior tibial tendons. Clavert et al. (2001) demonstrated significant changes in ultimate tensile failure and in Young's modulus in long head biceps tendons. Hirpara et al. (2008) and Smith et al. (1996) could not demonstrate any differences between fresh and fresh-frozen porcine flexor tendons. Arnout et al. (2013) showed that several freezing-thawing cycles had no influence on biomechanical properties. In contrast, Huang et al. (2011) demonstrated that multiple freeze–thaw cycles weakened tendon allografts. The influence of freezing remains controversial and is currently unresolved, but the changes observed are possibly caused by loss of tendon substance and fluid that occurs with freezing compared to fresh living tissue. With regard to the ultimate load for the fresh-frozen and fresh tendon groups, the standard deviation for both groups exceeded 30% of the mean and displayed a rather large variance. As suggested by Giannini et al. (2008) this high variability possibly reflects the natural differences in the biophysical tendon properties between individuals, but the effects of storage cannot be entirely disregarded.

The wide availability of embalmed cadavers may overcome these problems, but it is critical that these specimens maintain similar biomechanical properties compared to fresh tissue. Hansen et al. (2009) has shown that formalin embalming increases Young's modulus and peak forces by 100%, suggesting that collagen cross-linking increases the mechanical strength of the tendon. Wilke reported an 80% decrease in range of motion for formalin fixed spinal specimens, confirming cadavers are not truly representative of *in vivo* conditions (Wilke et al., 1996). Soft-fix embalming techniques have the benefit of potentially reduced health risks while also maintaining the physical properties of cadavers so they remain more similar to those encountered *in vivo* (Anderson, 2006; Liao et al., 2015). Several researchers have suggested this technique better maintains normal tissue characteristics, and is well suited for surgical training and biomechanical testing (Anderson, 2006; Benkhadra et al., 2009; Eisma et al., 2013; Wilke et al., 2011). The Thiel embalming technique was developed as an alternative preservation technique that maintains tissue pliability, flexibility, and color (Thiel, 1992). The Thiel method is principally used in Europe and is less well known globally (Benkhadra et al., 2011). The main

barrier to its more widespread adoption appears to be that most publications regarding this method have been written in German (Benkhadra et al., 2011). Fessel et al. (2011) loaded Thiel preserved human digitorum profundus tendons to failure and observed a trend towards a reduced elastic modulus. They suggested this was possibly due to partial denaturing by boric acid, and concluded that Thiel fixation does not faithfully preserve the characteristics of fresh-frozen tendon.

Liao et al. (2015) investigated the stress strain curve of Thiel-embalmed peroneus brevis and longus, and Achilles tendons, and compared their results to published values for fresh non-embalmed tendons. They demonstrated that tendon elasticity was lower for the Thiel-embalmed tendons, but increased with strain rates. Verstraete et al. (2015) compared stress–strain behavior of fresh-frozen and Thiel embalmed Achilles tendons, and demonstrated Thiel-embalmed tendons displayed a significantly higher elastic modulus whether they were dried or not. Similar to the findings of Verstraete et al. (2015), we also demonstrated a significantly higher elastic modulus for Thiel-embalmed tendons. The reasons for these differences between Verstraete et al. (2015), and our results compared to the findings of Liao et al. (2015) and Fessel et al. (2011), are not clear. The time of exposure to the preservation fluid appears to play an important role, and the longer tissues are exposed the more crosslinking can occur (Verstraete et al., 2015). Verstraete et al. (2015) also suggested that the variability of tissue perfusion could explain the reported variability. Arterial perfusion of a cadaver possibly results in higher penetration of highly vascular structures, such as muscle, with lower concentrations in less vascular structures, such as bone and tendon (Verstraete et al., 2015). Moreover, storage times in bags or tanks could be an additional factor by allowing embalming fluid to interact with tissues, which affects biomechanical properties. Unless these factors are known, comparisons between studies are very difficult. Finally, any innate difference in tissue elasticity between individuals introduces further bias, and remains uncontrolled but must also be taken into consideration (Louis-Ugbo et al., 2004).

The results of our study demonstrated similar loads to failure for both the formalin-embalmed, Thiel-preserved, and fresh-frozen tendons. Chemically preserved tendons could therefore be a reasonable alternative for load to failure testing. Hansen reported that formalin results in collagen crosslinking and induces significant increases in tensile strength (Hansen et al., 2009). However, other researchers have shown that chemical preservation has no effect on tensile strength (Lee et al., 1989; Pereira et al., 1990), or even decreases strength (Lee et al., 1989). Similar to Thiel fixation, exposure time and tissue penetration may have influenced the biomechanical properties of formalin-embalmed tissue.

Young's modulus and load to failure was significantly lower when comparing the fresh tendon group to the fresh-frozen and embalmed specimens. Relative to fresh specimens, for fresh-frozen tendons, Young's modulus was almost doubled and the load to failure tripled. It is possible that the tendon quality was inferior due to the underlying pathology, and the tendon integrity of some specimens may have been compromised. Although all tendons appeared macroscopically intact, microscopic changes may have occurred and could have altered the biomechanical properties. However, it is more likely that the significant differences are not only a result of the potentially compromised tendon quality, but are also related to structural changes that were introduced by the freezing and embalming process. The strength of this study is that we have directly compared the biomechanical properties of four different preservation techniques under the same conditions using a standardised testing protocol. To the authors' knowledge, this is the first study comparing four modes of preservation using human tendons. Several authors (Clavert et al., 2001; Giannini et al., 2008; Hirpara et al., 2008; Benkhadra et al., 2011; Verstraete et al., 2015; Liao et al., 2015) have previously compared the biomechanical properties of cadaveric tendon specimens using either an animal model or fewer groups.

This study has several inherent limitations. As with many other laboratory studies, the low sample size limits the external validity of the study. However, the a priori sample size calculation indicated this study would be able to detect a 20% difference between groups, and the results indicate that the study was adequately powered. For example, Young's modulus was 33% different when comparing fresh-frozen and fresh specimens, and the load to failure was 23% different, but in each case these changes were not significant. The age differences between the non-preserved and embalmed groups were substantial, and may have introduced an element of selection bias. However, the influence of age on tendon properties has not yet been established with certainty. In fact, Stenroth et al. (1985) suggested that there were no differences in the mechanical properties of human Achilles' tendons between young and older athletes. They suggested older people compensate for lower tendon material properties by increasing the cross-sectional area (Stenroth et al., 1985). Nakagawa et al. (1996) could not identify any age related changes in the biomechanical properties of Achilles' tendons in rabbits, but instead demonstrated variation in the tangent modulus of the mature tendon suggesting differences in elongation at failure. In contrast, a more recent study (Vafek et al., 2018) reported a significantly lower ultimate failure load, but no differences of Young's modulus, between young and old animals. Uncontrolled and unknown variability with the embalming process, and the uncontrolled length of the embalming period may have also influenced tissue elasticity.

5. Conclusions

The results of this biomechanical study suggest that both Thiel-preserved and formalin-embalmed long head of biceps tendons and fresh-frozen tendons have a similar load to failure. Either Thiel-preserved or formalin-embalmed tendon could therefore be considered reasonable alternatives for load to failure studies. However, Young's modulus for chemically preserved tendons was significantly stiffer than fresh or fresh-frozen specimens, and these methods might be less suitable alternatives when viscoelastic properties are being investigated.

Authorship contributions

Erik Hohmann (EH), Natalie Keough (NK), Vaida Glatt (VG), KevinTetsworth (KT), Reinhold Putz (RP) and Andreas Imhoff (AI). EH, RP, AI conceived and designed the study, NK, EH collected data, EH, NK, VG and KT wrote the paper, EH and NK performed the statistical analysis, EH, NK, VG and KT critically revised the paper; all authors gave final approval of the paper.

Acknowledgements

The authors thank the individuals and families whose selfless donation of bodies for research purposes made this study possible. All persons whose bodies were used in this study should be credited for the scientific achievements presented here that are intended to benefit the whole humanity.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.aanat.2018.05.002.

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