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THE EFFECT OF THIEL EMBALMING OR DEHYDRATION ON BIOMECHANICAL PROPERTIES OF TENDONS, AS COMPARED TO FRESH FROZEN TENDONS.

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Table of contents

1. ABSTRACT

1.1 English

Introduction:

Biomechanical research and orthopaedic training is often carried out on human cadavers. Because of the problem of rapid post-mortem decay, these cadavers are usually frozen or embalmed. The embalming method according to dr. Thiel is often praised for the preservation of natural colour, plasticity and texture of the cadavers. However, the normal in-vivo biomechanical properties could be influenced by Thiel embalming, while the purpose of biomechanical testing in general is precisely to elucidate the in vivo biomechanical properties. Therefore, further research is needed.

Furthermore, in most studies which examine the biomechanical properties of tendon and other human tissue, care is taken to preclude dehydration of the tissue. To our knowledge however, the precise effect of drying out of desiccation tissue on the biomechanical properties, has not yet been quantified.

Objectives:

The primary objective of this study is to examine whether Thiel embalming alters the biomechanical properties of Achilles tendons compared with thawed fresh frozen specimens.

The secondary objective of this study is the investigation of the influence of dehydration on biomechanical properties of both thawed fresh frozen tendons and Thiel embalmed tendons.

Materials & methods:

Both Achilles tendons from seven cadavers were used in this study. Each time one of the Achilles tendons was frozen and the other was Thiel embalmed, in order to enable a paired comparison of the biomechanical properties. The tendons were loaded gradually in a specifically designed clamping device. Elongation of each tendon was measured with 'Differential Variable Reluctance Transducers' and 'Digital Image Correlation'. The cross-sectional area was measured with a calliper and with microCT . This allowed calculation of the modulus of the toe-region and the linear region of each tendon via the stress-strain curve. The primary objective was obtained by comparing the moduli of the fresh frozen group with the Thiel embalmed group. The secondary objective was obtained by comparing the moduli of

the fresh frozen non-dried group with the fresh frozen dried group and also by comparing the Thiel embalmed non-dried group with the Thiel embalmed dried group.

Results:

1. Primary objective:
    - *Modulus of Thiel embalmed tendons compared with the modulus of fresh frozen Achilles tendons*: The modulus of the toe-region does not significantly differ between the two groups (P=0,249), but the general trend is that the modulus of the toe-region of Thiel embalmed tendons is higher. The linear region of Thiel embalmed tendons is significantly stiffer (higher elastic modulus) than fresh frozen tendons (P=0,046).
2. Secondary objective:
    - *Modulus of non-dried (not dehydrated) fresh frozen Achilles tendons compared with the modulus of dried (dehydrated) fresh frozen Achilles tendons:* The modulus of the toe-region does not significantly differ between the two groups (P=0,249), but the general trend is that the modulus of the toe-region of dried fresh frozen tendons is higher than non-dried fresh frozen tendons. The linear region of the dried fresh frozen tendons is significantly stiffer than the non-dried fresh frozen tendons (P=0,046).
    - *Modulus of non-dried Thiel embalmed Achilles tendons compared with the modulus of dried Thiel embalmed Achilles tendons:* Neither the modulus of the toe-region (P=0,225) nor the modulus of the linear region (P=0,225) are significantly different. No general trends were visible.

Conclusion:

This study demonstrates that Thiel embalming significantly alters the biomechanical properties of tendons and is thus not suitable for biomechanical testing. The results of this study, demonstrating an increase of the modulus of the linear portion of the stress-strain curve after Thiel embalming, are in contrast with other studies that mention a decrease of the modulus of several tissues after Thiel embalming.

Concerning the effect of dehydration on biomechanical properties of human tissue, it was demonstrated that exposure of thawed fresh frozen human tissue to room conditions for two hours without moistening, significantly alters the biomechanical properties. More specifically,

an increasing of the modulus of the linear portion can be seen. It can thus be stated that in studies where moistening of tissue is impossible for longer than two hours, results should be interpreted with caution. A significant alteration of biomechanical properties of Thiel embalmed tendons when exposed to room conditions could not be demonstrated.

1.2 Nederlands

Introductie:

Biomechanisch onderzoek en orthopedische training worden vaak uitgevoerd op pezen van menselijke kadavers. Het probleem met menselijke kadavers is het feit dat ze post-mortem niet lang kunnen bewaard worden. Daarom worden deze kadavers vaak ingevroren of gebalsemd. De balseming methode beschreven door dr. Thiel wordt vaak geprezen omwille van het behouden van de natuurlijke kleur, plasticiteit en textuur van het gebalsemde lichaam. Deze bewaringstechniek kan echter de biomechanische eigenschappen beïnvloeden, terwijl het doel van biomechanisch onderzoek net is de normale in-vivo biomechanische eigenschappen te achterhalen.

Hiernaast wordt in de meeste studies die de biomechanische eigenschappen van pezen of ander humaan weefsel onderzoeken veel aandacht geschonken aan preventie van uitdroging. In de literatuur wordt er echter nergens gekwantificeerd wat precies het effect van uitdroging is op de biomechanische eigenschappen van pezen en ander humaan weefsel.

Objectieven:

Het primaire doel van deze studie is onderzoeken of Thiel-balseming de biomechanische eigenschappen van achillespezen wijzigt in vergelijking met ontdooide diepgevroren achillespezen.

Het secundaire doel van deze studie is de invloed van uitdroging op de biomechanische eigenschappen van zowel ingevroren als Thiel-gebalsemde achillespezen te kwantificeren.

Materialen & methoden:

Van zeven kadavers werden beide achillespezen gebruikt. Telkens werd één van de pezen ingevroren en werd de andere Thiel-gebalsemd. Daardoor was het mogelijk om in deze studie een gepaarde vergelijking te doen van beide achillespezen van ieder kadaver. De achillespezen werden gradueel belast in een specifiek ontworpen klemsysteem. Elongatie van de achillespezen werd gemeten met ‘Differential Variable Reluctance Transducers’ en ‘Digital Image Correlation’. De doorsnedes van de achillespezen werden gemeten met een schuifmeter en met een micro-CT. Dit alles maakte het mogelijk om de modulus van de toe-regio en lineaire regio te berekenen via een bilineaire fit, toegepast op de stress-strain curves. De primaire onderzoeksvraag werd onderzocht door het vergelijken van de moduli van de ingevroren groep met deze van de Thiel-gebalsemde groep en dit zowel voor de toe als voor

de lineaire regio van de curve. De secundaire onderzoeksvraag werd onderzocht door het vergelijken van de moduli van de niet-uitgedroogde, ingevroren groep met deze van de uitgedroogde, ingevroren groep. Ook de niet-uitgedroogde Thiel-gebalsemde groep werd vergeleken met de uitgedroogde Thiel-gebalsemde groep.

Resultaten:

1. Primair doel:
    - *Vergelijking van de modulus van de ontdooide diepgevroren achillespezen met deze van de Thiel-gebalsemde achillespezen*: De toe-regio is niet significant verschillend (P=0.249), maar de algemene trend is dat de modulus van de toe-regio van Thiel-gebalsemde pezen groter is. De lineaire regio van Thiel-gebalsemde achillespezen is significant stijver (grotere elasticiteitsmodulus) dan de ingevroren achillespezen (P=0.046).
2. Secundair doel:
    - *Vergelijking van de modulus van de niet-uitgedroogde, diepgevroren achillespezen met de modulus van uitgedroogde, diepgevroren achillespezen*: De toe-regio is niet significant verschillend (P=0.345), maar de algemene trend is dat de modulus van de toe-regio van uitgedroogde, ingevroren achillespezen groter is. De lineaire regio van de uitgedroogde, ingevroren achillespezen is significant stijver dan de niet-uitgedroogde, ingevroren achillespezen (P=0.046)
    - *Vergelijking van de modulus van de niet-uitgedroogde, Thiel-gebalsemde achillespezen met deze van uitgedroogde, Thiel-gebalsemde achillespezen:* Noch de modulus van de toe-regio (P=0.225), noch de modulus van de lineaire regio (P=0.225) zijn significant verschillend. Er zijn geen algemene trends aantoonbaar.

Conclusie:

Deze studie toont aan dat Thiel balseming de biomechanische eigenschappen van achillespezen significant wijzigt en dat Thiel gebalsemd weefsel dus niet geschikt is voor biomechanische testen. De resultaten van deze studie, waarin een verhoging van de modulus van het lineaire gedeelte van de ‘stress-strain’ curve kan gezien worden na Thiel-balseming, staan in contrast met andere studies die een daling van de modulus van allerlei weefsels na Thiel balseming beschrijven.

Betreffende het effect van uitdroging op de biomechanische eigenschappen van menselijk weefsel, toonden de resultaten van deze studie aan dat blootstelling gedurende twee uur van ontdooid diepgevroren menselijk weefsel aan kamerlucht zonder bevochtiging, de biomechanische eigenschappen significant wijzigt. Meer specifiek kan een stijging van de elastische modulus worden waargenomen. In studies waar de bevochtiging van weefsel onmogelijk is voor langer dan 2 uur, moeten de resultaten bijgevolg worden geïnterpreteerd met de nodige omzichtigheid. Na blootstelling van Thiel gebalsemde pezen aan de kamerlucht kon geen significante verandering van de biomechanische eigenschappen worden aangetoond.

2. INTRODUCTION

2.1 General introduction

Orthopedic training and biomechanical research are largely dependent on the availability of human cadavers. The problem with fresh or thawed fresh-frozen human cadavers is the fact that they start to decay rapidly post-mortem and that they are very expensive. Because of the need for undemanding and decent preservation of cadavers for long periods of time and the reduction of the biological risk, human cadaveric specimens are often embalmed (3).

The classical embalming methods are based on fluids containing a high concentration of formaldehyde or glutaraldehyde. These methods have proven to be very effective to stop the degeneration process, to be strongly disinfectant and to preserve the overall histological properties of human tissue (3). Yet there are important drawbacks to this classical embalming technique: besides the fact that high levels of aldehydes are toxic (4), most studies suggest that aldehydes alter the properties of collagen tissue (5-8). The embalmed tissue becomes stiffer and more brittle in respect to fresh or fresh frozen tissue (5;7;9), due to intermolecular cross-linking of proteoglycan monomers (e.g. of collagen) (5).

The above mentioned facts imply that biomechanical testing on formalin-fixed specimens are not representative for the in vivo properties, while the purpose of biomechanical testing in general is precisely to elucidate the in vivo biomechanical properties. Besides, the increased stiffness makes formalin/gluteraldehyde embalmed tissue unsuitable for surgical or student anatomical training (10).

Because of these concerns there is increasing interest in alternative preservation methods. Especially embalming according to the Thiel embalming procedure has gained interest. The Thiel embalming technique is developed in Austria and is firstly reported by dr. W. Thiel in 1992. The embalming fluids are based on water, glycol, boric acid and various salts. Thiel embalmed specimen are considered to be at least as good as fresh-frozen material with preservation of natural colour, plasticity and texture and without the health and durability issues (10;11).

Taking into account all the advantages of Thiel embalming, it would be very convenient to use Thiel embalmed tissue for biomechanical studies. However, concerning the exact biomechanical properties, it is not evident that Thiel embalmed tissue is representative for in

vivo conditions. The primary objective of this study was therefore to examine whether Thiel embalming alters the biomechanical properties of Achilles tendons compared to fresh frozen specimens (which are representative for the in vivo tissue properties (12)), in order to confirm or refute the suitability of Thiel embalmed tendons in biomechanical studies.

The secondary objective of this study is the investigation of the influence of dehydration on biomechanical properties of thawed fresh frozen and Thiel embalmed tendons. In most studies examining the biomechanical properties of tendons and other human tissue, care is taken to preclude dehydration of the tissue. However, to our knowledge, the precise effect of drying out on the biomechanical properties of tissues, has not yet been quantified. In some biomechanical study settings, continuous moistening of the studied tissue is not possible. Therefore, it is interesting to investigate to what extent results of such studies are reliable and in accordance with in vivo biomechanical properties. In addition it would be also opportune to know if biomechanical properties of Thiel embalmed tissue are susceptible to exposure to room conditions and the lack of moistening as well.

2.2 Biomechanical properties

In order to be able to compare the biomechanical properties of the tendons, basic values are needed to quantify these biomechanical properties. These values can be derived from the tendon's specific stress-strain curve. During loading, tendons are subjected to different stresses (Force/cross-sectional area) that cause certain strains (amount of deformation in %) in the specimens. This relationship between paired stresses and strains of a particular specimen, can be plotted in the specimen's specific stress-strain curve. These curves reveal many of the properties of a material.

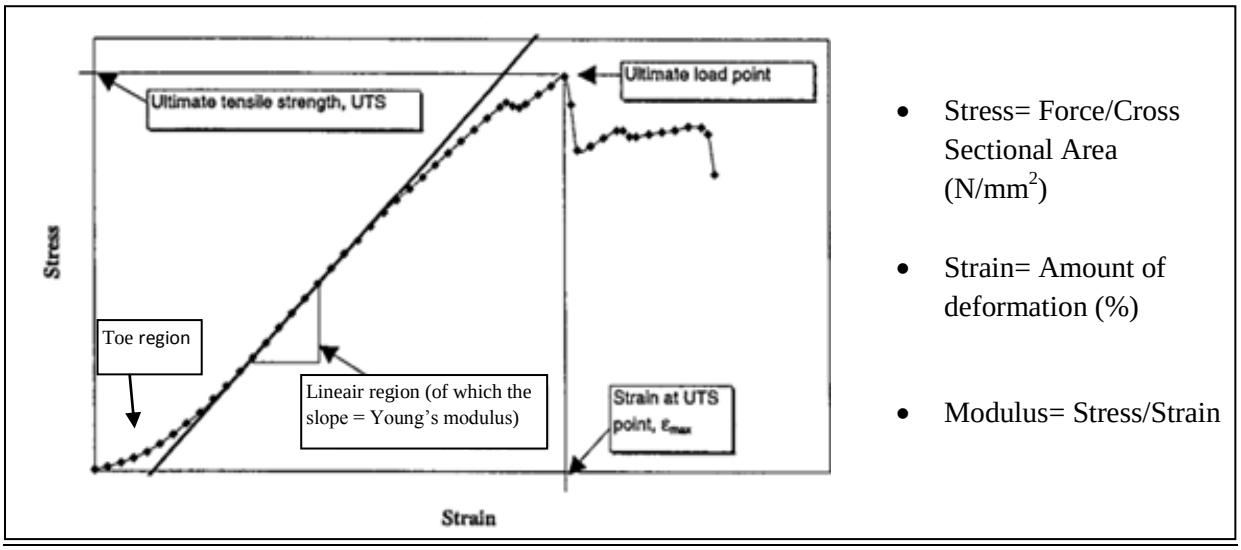


Fig 1: Typical representation of the shape of a tensile stress-versus-tensile strain curve. The curve displays three regions: a low-modulus toe zone; a linear zone of the post-toe section over which the tangent to the curve is essentially invariant; and a yield and failure zone where the modulus (the tangent to the curve) first drops and substantially decreases before fracture. (2)

As illustrated in figure 1, the stress-strain curve of soft tissue (e.g. tendons) begins with a non-linear toe-region at low loads. This so called toe-region is characterized by a large increase in strain with increasing stress. Hereafter, the curve proceeds to a linear region with an approximately constant modulus of elasticity when load increases (13). This modulus of elasticity is also referred to as Young's modulus (YM). It is defined as the slope of the stress–strain curve in the elastic (linear) deformation region and describes a specimen's tendency to be deformed elastically (i.e., non-permanently) when a is applied to it. As such, a stiffer material will have a higher elastic modulus. Because the terms stress and strain already comprehend the cross-sectional area and the length of the specimen, the modulus characterizes a property which is independent of these values. The modulus thus represents the stiffness normalized by its length and cross-sectional area (CSA) and is a dimensionless measure of tendon stiffness. Additionally, the yield point (stress and strain values when the tendon begins to fail and deforms irreversibly) and ultimate stress and strain (rupture of the tendon) can be determined from the stress-strain curve. All above mentioned values can be used to determine the biomechanical properties of a tendon or other material.

2.3 Relevant studies in literature

Concerning literature relevant to our study objectives, only few studies can be found. To our knowledge, only three studies have been performed to examine the alteration of biomechanical properties of tissue after Thiel embalming (9;14;15). Only Fessel et al examined the influence of Thiel embalming on tendons: the influence on biomechanical properties of flexor digitorum tendons (FDP) and fascicles of rat tail tendons. The researchers concluded that Thiel embalmed FDP tendons were characterized by lower failure stresses than fresh frozen tendons and showed trends towards a reduced elastic modulus (although the results were not significant). This observation of a trend towards a lower modulus of Thiel embalmed tissue is confirmed by two other studies on spinal motion (9) and bone specimens (15).

An exact explanation for this alteration of biomechanical properties remains unknown (14;16). Benkhadra et al have tried to formulate an explanation by histological investigation of Thiel embalmed tendon. According to their report, the histology of collagen fibres of tendon was identical in both Thiel embalmed and fresh tissue (16).

Concerning quantification of the effect on the biomechanical properties when human tissue is exposed to room conditions, no studies have been found.

2.4 Research questions

Two main research questions are formulated in this study:

A. 1) Does Thiel embalming of human tendon tissue alters the biomechanical properties in comparison to thawed fresh frozen tendons?
   2) Does Thiel embalming alters the histological properties of Achilles tendon?
B. To which extent are biomechanical properties of both thawed fresh frozen tendons and Thiel embalmed tendons influenced by exposure to room conditions (room temperature, room humidity,..) and the resultant dehydration and degeneration?

3. MATERIALS & METHODS

3.1 Dissection and storage

Achilles tendons of human cadavers were obtained from the anatomy lab of the Ledeganck, Faculty of Medicine of Ghent University. There were six female cadavers and one male cadaver. The age of the cadavers ranged from 45 to 89 years with a mean age of 77 years. The cadavers arrived at the anatomy lab within a few hours post-mortem. Both Achilles tendons from each cadaver were harvested. The first Achilles tendon (with no preference for the left or right tendon) was prelevated rapidly post-mortem. Tendons were prelevated with a part of the calcaneus on the distal side and a part of the gastrocnemius muscle on the other side still attached.

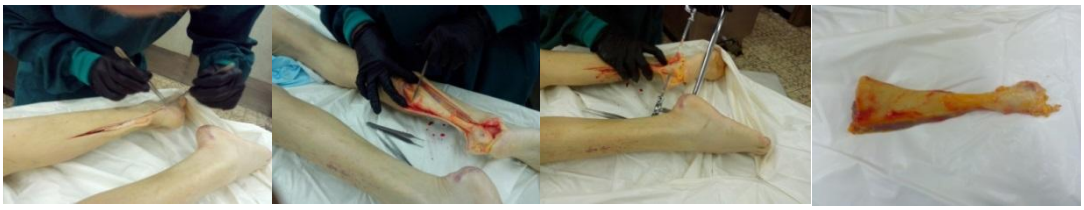


Fig 2: Illustration of the prelevation of the Achilles tendons.

The Achilles tendon was subsequently wrapped in cloths soaked with physiological fluid (0.9%NaCl) and immediately placed in a freezer at a temperature of -20°C. The tendon was frozen for a duration of minimum seven days up to maximum fourteen days, after which the tests were immediately performed .

After the first tendon was removed, the body was embalmed as described below. This procedure is in accordance with the Thiel embalming procedure as described in the original articles of dr. Thiel (3).The cadavers were perfused ultimately two days after death in the femoral artery with a solution containing 14,300 ml of solution A and 500 ml of solution B. Both solutions are prepared beforehand in large quantities and are mixed in the correct proportions just before perfusion, together with 700 g of sodium sulfite as well as 300 ml of formalin.

Solution A contains ethyleneglycol (190 ml/l), ammonium nitrate (126 g/l), boric acid (19 g/l), potassium nitrate (32 g/l) and water (633 ml/l). Solution B contains ethylene glycol (910 ml/l) and 4-chloro-3-methylphenol (90 g/l).

Subsequently, the cadavers were immersed for approximately six weeks in a solution containing boric acid (21,6 g/l), ethylene glycol (71,9 ml/l), ammonium nitrate (71,9 g/l), potassium nitrate (36 g/l), solution B (14,4 ml/l), sodium sulfite (50 g/l), formalin (14,4 ml/l)

and water (720 ml/l) (See also ‘attachment 2’ for a table of the composition of the different solutions.).

When this embalming procedure finished after exactly six weeks, the second (contralateral) Achilles tendon was prelevated. This tendon was then wrapped in cloths soaked in Thiel embalming fluid and immediately stored in a refrigerator with a temperature of 4°C for a maximum duration of fourteen days before testing.

3.2 Preparation & Clamping

During the tests, dehydration of the tendon was prevented at any time with cloths soaked with physiological fluid (thawed fresh frozen tendons) or Thiel embalming fluid (Thiel embalmed tendons). Both the fresh frozen tendons and the Thiel embalmed tendons were first prepared by careful removing the epi- and perithenon of the tendons. In fresh frozen tendons this occurred after thawing (approximately 60 min) at room temperature. A mark was made on the tendon with a surgical marker at the height of the most cranial level of the calcaneus. Then the attached bone-block was carefully removed.

The tendon was subsequently clamped in custom-made clamps for large tendons. The positioning of the tendon between the clamps was always identical, using the applied mark as target point. The length of the tendon between the clamps was 4 cm on average, ranging from 3,5 cm to 4,5 cm.

The clamping device itself consisted of two major parts for clamping both the caudal and cranial part of the tendon. On the major plates, the tendon was clamped with the aid of eight screws and two minor metal plates that had a serrated polymer toothed rack on the inside. These serrations of each metal plate fitted perfectly into one another in such way that the clamped portion of the tendon was clamped maximally. The screws were tightened with a force of 7N per screw. The two major plates were connected to each other via two pipes which prevented the rotation of the major plates relative to each other. Differential Variable Reluctance Transducers (DVRT’s) were mounted in the frame next to the tendon.

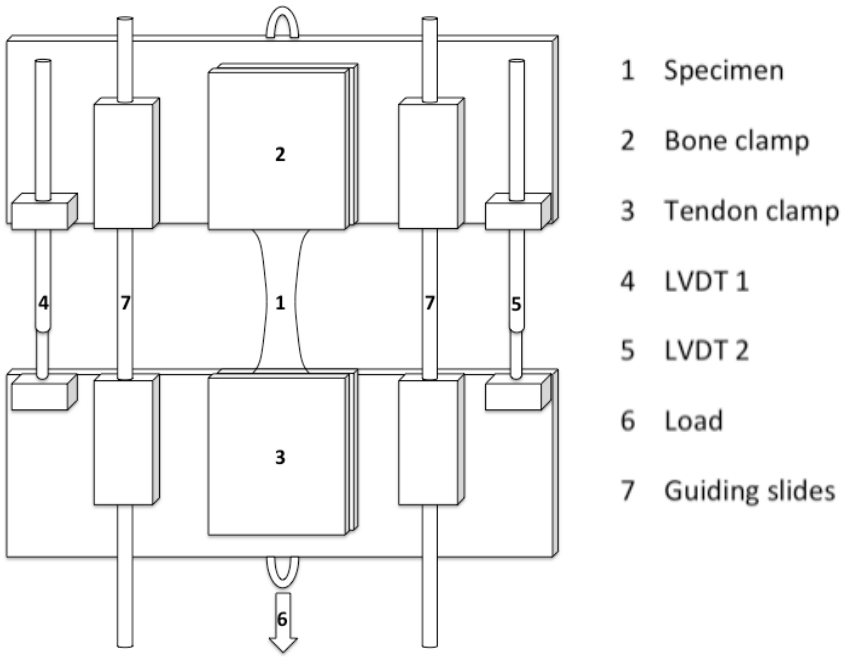


Fig 3: Illustration of the clamping setup.

3.3 Testing and Determination of Biomechanical Properties

3.3.1 General biomechanical properties

On the moment that the clamping has been executed, the gradual loading of the tendons to determine the biomechanical properties begins. During loading, the tendons are subjected to different stresses that cause certain strains in the specimens. This relationship between paired stresses and strains of each tendon was plotted in the specimen's specific stress-strain curve (see introduction).

In this study, the main focus was the modulus as most important determinant of biomechanical properties. The elastic modulus was calculated on the basis of the tangent to the linear portion of the specific stress-strain curve.

In addition, an approximate value of the modulus for the toe-region was computed as well (an explanation why characterization of the toe-region properties is important can be found in 'discussion'). To distinguish the toe-region (low-strain properties) from the linear region (high-strain properties), the transition point on the stress–strain curve between those regions was determined. The whole stress-strain curve was therefore fit to a bilinear constitutive model, using the least squares method. Using this model, the modulus of the toe-region and linear region can be determined as well. These processes were executed in an application that was specially programmed for this purpose in excel (Microsoft Excel 2010). The formula for the bilinear fit was:

$$\sigma = E_0\varepsilon \quad (\text{where} \varepsilon \leq \varepsilon^*)$$

$$\sigma = E(\varepsilon - \varepsilon^*) + E_0\varepsilon^* \quad (\text{where } \varepsilon > \varepsilon^*)$$

(σ = stress, ε =associated strain, ε^* =strain transition point between toe-region and linear region (transition strain), E0 =modulus of elasticity of the toe region and E = modulus of elasticity of the linear region)

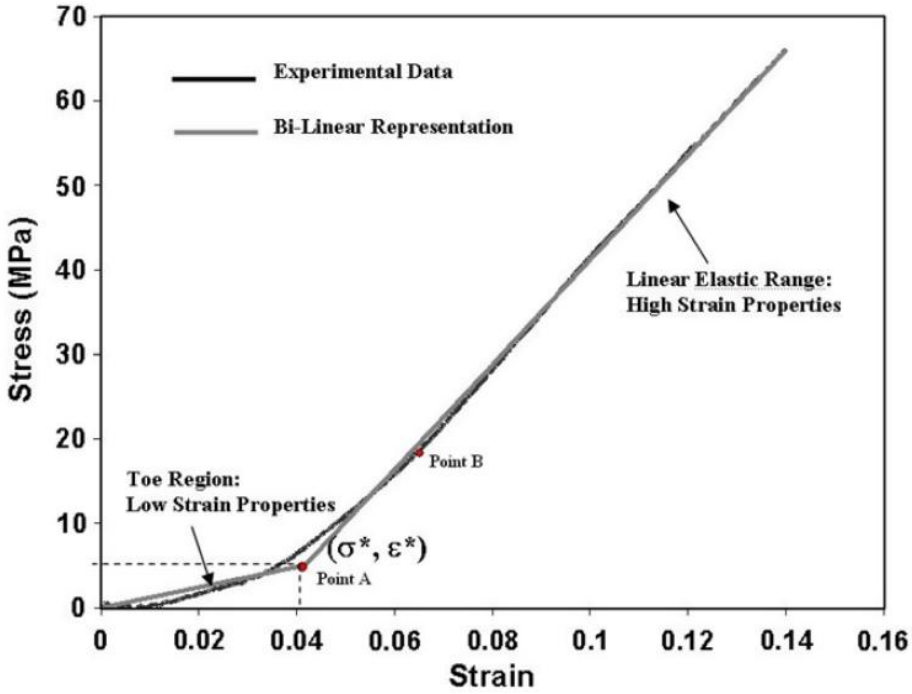


Fig 4: On the basis of the experimental data and the application of the bilinear constitutive model, a bilinear representation was made. This way, the transition point between toe-region and linear region and the modulus of the toe-region and linear region can be deducted (17).

In order to draft the stress-strain curve of each tendon and to determine the linear modulus and the modulus of the toe-region, several data (such as Cross-Sectional Area (CSA), deformation per load, ...) are required. The devices and methods used to acquire these data are elucidated below (**3.3.2** till **3.3.5**).

Each test was executed twice for each tendon: the first time immediately after clamping and the second time after 2-3 hours exposure to room conditions without hydration (while still being clamped).

3.3.2 Loads & preloading

In earlier experiments, some slippage out of the clamps was observed. Slip was largely reduced by preloading each Achilles tendon with 350N for two minutes (this reconciles with a mean strain of 6±1 %). During preloading, extra attention was paid to keep the tendon well moistened.

After preloading the experiment started with a load of 33N (the load being exercised by the weight of the caudal major plate and minor plates of our clamping system). Thereafter extra load of 17N was applied by attaching the weight carrier. Subsequently, the following weights were added stepwise: 25N, 50N, 75N, 100N, 125N, 150N, 200N, 250N, 300N, 400N, 500N, 600N, 700N & 800N. The reason why an increase in weight by only 25N was firstly admitted, is because the toe-region is more visible by a small increase in load.

The displacement was measured after each increase in load with the aid of DVRT's (in four tests the DIC (digital image correlation) technique was used in addition). Hence we performed a static measurement.

Fig 5: Loaded testing setup.

3.3.3 Differential Variable Reluctance Transducers (DVRT's)

In all tests, DVRT's were used to measure the displacement in function of the load applied on the tendon. DVRT's are little devices that perceive uni-axial displacement by changes in magnetic field. The displacement is displayed as a difference in voltage. Using a specific calibration formula, this difference can be converted to a difference in distance. The used DVRT's had an accuracy of 1µm. The DVRT's were mounted in the clamping device and when the loads were removed after testing, the DVRT's displayed a value that differed slightly from the unloaded starting-point before the test. This difference was defined as the slippage out of the grips which was compared between all the tested groups (fresh frozen non-dried, fresh frozen dried, Thiel embalmed non-dried, Thiel embalmed dried) with the Wilcoxon signed ranks test. The values of slippage and the statistical comparisons are displayed in addendum 4.

3.3.4 Digital Image Correlation

DIC was also used as a measuring method together with DVRT's on four Achilles tendons. DIC is a relatively novel technique which is suitable for quantification of local mid substance strains, multi-axial strains and in the current study, to quantify slippage out of the grips (18). Briefly, DIC is an optical method that employs tracking and image registration techniques for accurate 2D and 3D measurements of displacement in images. In most DIC applications, a high contrast speckle pattern is applied onto the surface of the sample and observed by charge-coupled device (CCD) cameras (19). If there is a deformation of the substance (e.g. after the application of loads), individual spots on the surface of the specimen can be tracked on the images. In the current study, a white speckle pattern was applied with aerosol paint after clamping of the tendons. Besides the aerosol paint, a toothbrush to make the speckle pattern was sometimes used. The toothbrush was remarkably easier to use than the aerosol paint. Prior to the speckling, the tendon specimens were colored with methylene blue to supply a sufficient contrast between the speckle pattern and the background (the higher the contrast, the better the accuracy). Methylene blue penetrates the tissue without leaving a coat on the surface of the tendons (Fig 6). A speckle pattern was also applied to the clamps in order to be able to quantify the slippage out of the clamps.

The 'movement' of the speckles was than analysed using specialist software (VIC3D 2009, Correlated Solutions Inc.) to determine the local displacements. Concretely, software programs achieve this by dividing the area of interest on the images into a number of unique correlation areas, or 'facets', which typically contain a square subset of pixels. The determination of the displacement then relies on the maximization of a correlation coefficient that is determined by examining the pixel intensity array subsets on two or more corresponding images. The strains on the surface can then be derived from the displacement fields (19;20).

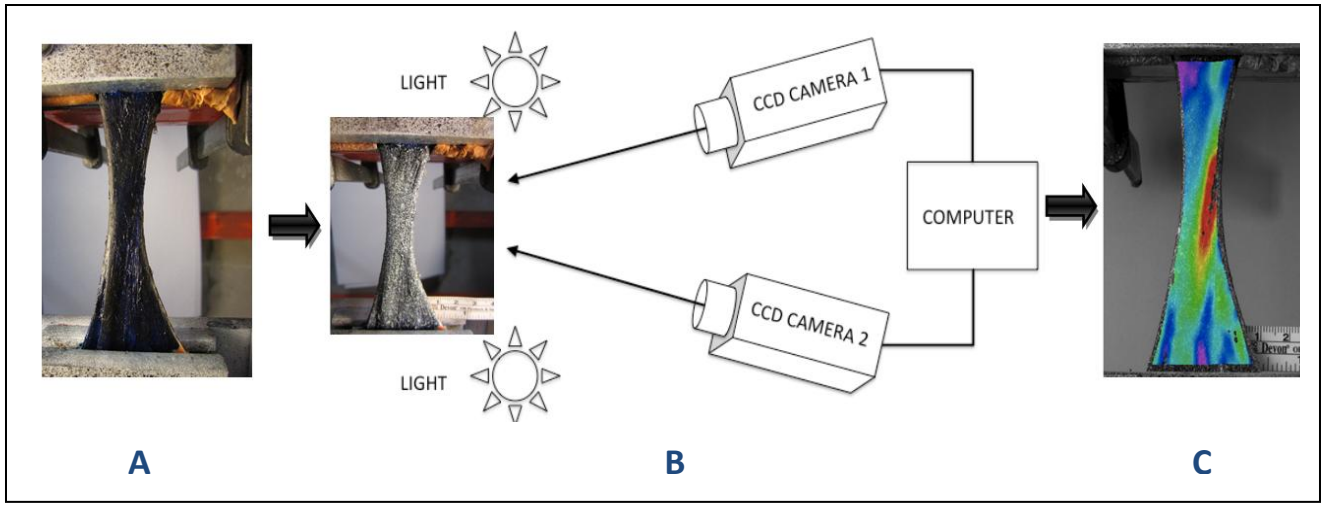
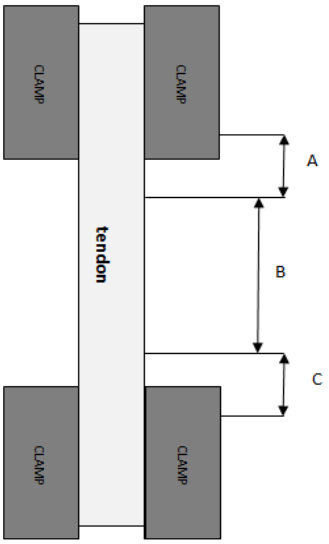


Fig 6: Achilles tendon dyed with methylene blue (A), then speckled and afterwards loaded while being monitored by CCD camera's (B). Computer software (VIC3D 2009, Correlated Solutions Inc.) analyses the strain patterns with each loading (C).

As mentioned above, the main purpose of using DIC in this study was to quantify slippage out of the grips. To do so, the mean grip-grip displacement (A+B+C) is compared to the mean tendon displacement (B) and is defined as the X-value. This X-value gives an indication of the amount of observed displacement that is actually caused by displacement of the tendon and not caused by slippage out of the grips. This X-value overestimates the contribution of slip to the overall displacement value because A and C do not solely represent slippage out of the grips. Indeed a part of the A en C value is caused by actual displacement of the small piece of tendon in A and C. The X-values and the statistical comparison are displayed in addendum 4.



$$X = \frac{B}{A + B + C}$$

A : mean displacement of the A-section (mm)

B : mean displacement of the B-section (mm)

C : mean displacement of the C-section (mm)

X: ratio of mean B-section displacement compared to total displacement.

Fig 7: Lateral view of clamped-tendon.

3.3.5 Area measuring

Two different methods were used to determine the cross-sectional area of the tendon. First, the mean cross-sectional area was measured with the aid of a caliper. To do so, we measured the width and thickness at three different levels of the tendon between the clamps: at the level of the caudal, middle and cranial part of the tendon. Based on the width and thickness, each area was calculated considering the area to be elliptical (It is most truthful to assume an ellipse shape when determining the cross-sectional area manually) (36). The average of these three values was calculated and this average cross-sectional area was used for calculation of the stress (load/area) .

Secondly, a micro-CT (μCT) was taken from each tendon after the test. The resulting images were subsequently analysed with the software programme MIMICS. MIMICS is a software programme developed by Materialise (Technologielaan 15 3001 Leuven, Belgium) for medical processing of 3D medical images, resulting in accurate 3D models. In MIMICS, the mean cross-sectional area could be measured with the aid of the software much more accurately at the same three different levels. The overall mean cross-sectional area of each tendon was calculated by taking the average of these three values. Besides, a value for the mean CSA was also calculated by averaging the area of 1024 axial slices of the tendon in MIMICS.

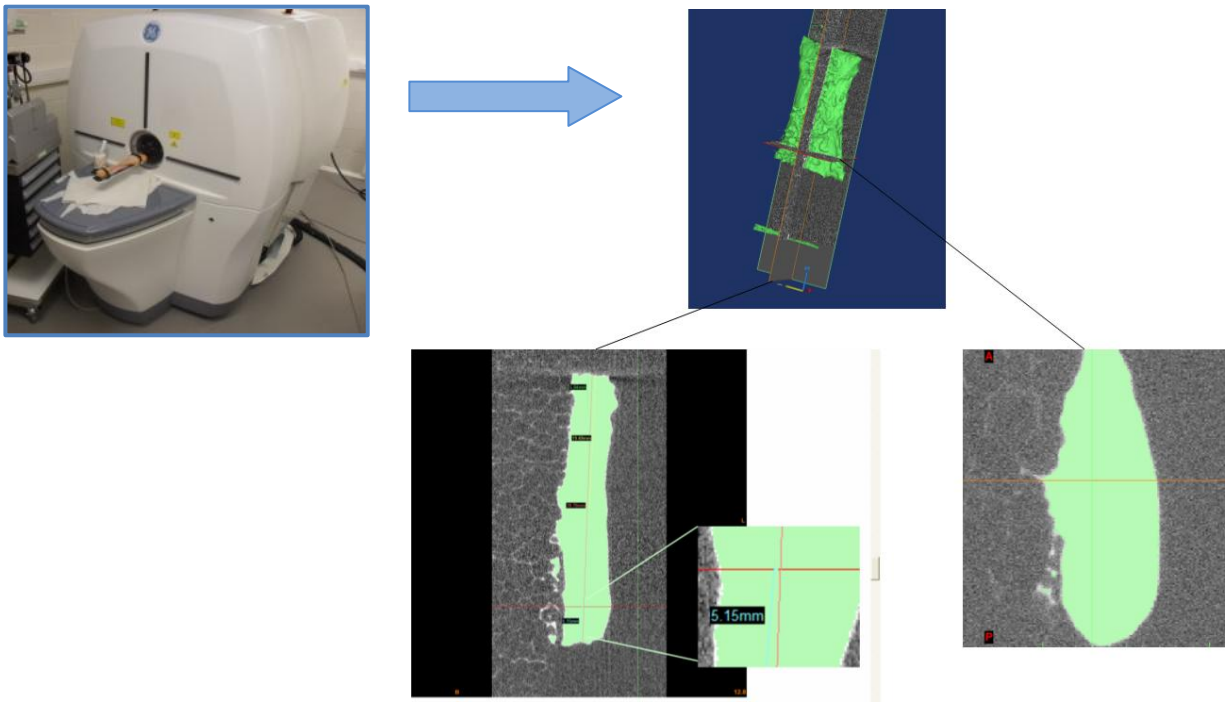


Fig 8: CT-data analysis using MIMICS software. Measurements in the sagittal plane were done to recover the three levels where areas were measured with the calliper. At these levels the area's in the axial plane were calculated by the software.

We compared the CSA measured with the calliper before and after it was dried, of each tendon. The comparison revealed that there is no significant difference (Wilcoxon matched pair rank test, P=0,484) between the non-dried (fresh frozen and Thiel) and dried (fresh frozen and Thiel) tendons. Consequently, the CSA measured with µCT (the CSA of the dried tendons) is also representative for the CSA of the non-dried tendons. Therefore the CSA measured with µCT was used for all further calculations in this study.

The mean area of the three marked axial CT slices was also compared to the value for the mean CSA calculated by averaging the area of all (1024) axial slices of the tendon. These values did not differ significantly (Wilcoxon signed ranks test: P=0.753). The mean difference between those two values was only 0.68 mm^2 (±12.18SD) . Thus it can be stated that measuring the CSA of only three slices (top, middle, bottom) is a good approximation of the overall CSA.

3.4 Statistics

To compare the moduli of all sample pairs, the statistical software IBM SPSS Statistics 20 was used. The population of moduli was not normally distributed (e.g. figure 9). Therefore non-parametric statistical tests (Wilcoxon signed ranks test) were used to compare the paired samples. Additionally, the paired Kruskall-Wallis test was performed and graphically visualized by a box plot. All statistical processing was reviewed by Professor George Van Maele (Department medical informatics and statistics Ghent University).

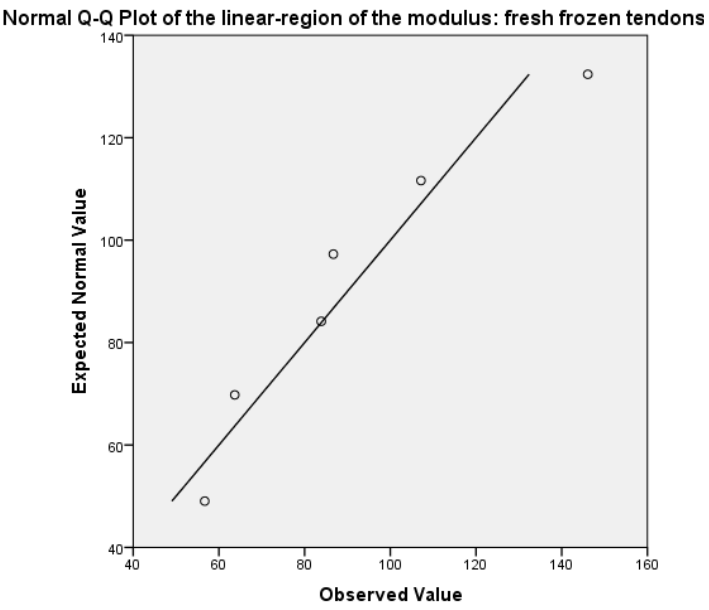


Fig 9: Q-Q plot of the modulus values. Note that these values are not completely normally distributed.

3.4 Histological analysis

A microscopic investigation of a fresh frozen and Thiel embalmed Achilles tendon was performed to search for differences that might explain the difference in biomechanical properties between fresh frozen and Thiel embalmed tendons.

Biopsies of a fresh frozen and a Thiel embalmed tendon were taken with a scalpel before testing. In both tendons, two biopsies were conducted: one in the area that was clamped afterwards in the lower grip (near the calcaneal insertion) and one in the area that was clamped afterwards in the upper grip (alongside the gastrocnemius muscle). All samples were dehydrated through progressive increasing concentrations of ethanol and thereafter embedded in paraffin (more details in addendum 1 "embedding in paraffin"). When the embedment in paraffin was finished, the samples were sectioned and standard hematoxylin and eosin stain (H&E) was applied (more details in addendum 2 "Haematoxylline-eosinestaining"). Furthermore, a trichrome staining was applied which makes it possible to visualize connective tissue in more detail. The slides were evaluated with light microscopy by R. Cornelissen (PhD, head of the lab of Histology, Department Basic medical sciences - Faculty of Medicine, UGent).

3.5 Examination of the research questions

In conclusion, the research questions were examined as follows:

A.1 Comparison between biomechanical properties of fresh frozen and Thiel embalmed tendons was made by performing a paired comparison of the left and right Achilles tendon of the same body of which one was frozen and the other Thiel embalmed. The tendons were loaded gradually and elongation of the tendons was measured with DVRT's and DIC. The cross-sectional area was measured with a calliper and micro-CT. The modulus of the toe-region and the linear region of each tendon was calculated via the stress-strain curve and compared with the contra lateral tendon using the Wilcoxon signed ranks test.

A.2 Influence of Thiel embalming on histological properties was examined by analysing biopsies of a fresh frozen and a Thiel embalmed tendon. All samples were embedded in paraffin, sectioned and stained with standard hematoxylin-eosin or trichrome staining. Afterwards the slides were evaluated with a light microscope.

B. The influence on thawed fresh frozen and Thiel embalmed tendons by exposure to room conditions was examined by making paired comparisons of the modulus of the toe-region and the linear region of each tendon before and after 2-3 hours exposure to room conditions.

4. Results

4.1 Comparison of biomechanical properties between fresh frozen and Thiel embalmed tendons

4.1.1 Toe-region

The modulus of the toe-region is not significantly different between fresh frozen and Thiel embalmed tendons (P=0,249). The general trend is that Thiel embalmed tendons are stiffer than fresh frozen tendons. The mean difference is 8,96 MPa ± 27,88 with a range of 80,17 MPa.

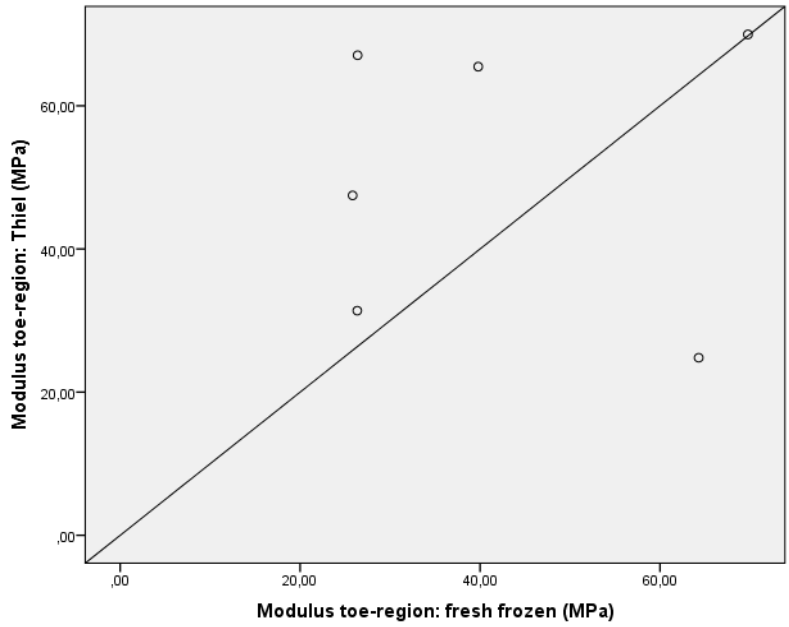


Fig 10: Scatter plot with line of identity. Modulus of toe-region of fresh frozen tendons vs. modulus of toe-region of Thiel embalmed tendons.

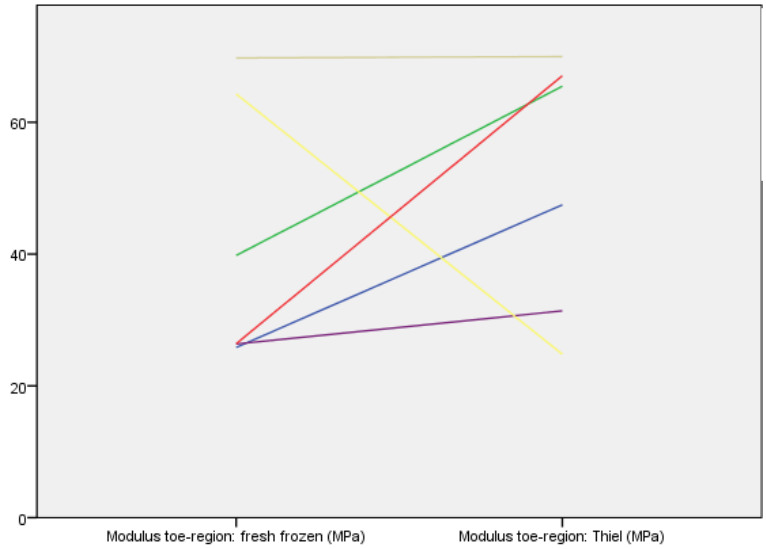


Fig 11: Line chart. Modulus of toe-region of fresh frozen tendons vs. modulus of the toe-region of Thiel embalmed tendons.

4.1.2 Linear region

In contrast to the toe-region, the modulus of the linear region is significantly different between fresh frozen and Thiel embalmed tendons (P=0,046). Thiel embalmed tendons have a greater modulus than fresh frozen tendons. The mean difference is 49,63 MPa ± 40,38 with a range of 124,96 MPa.

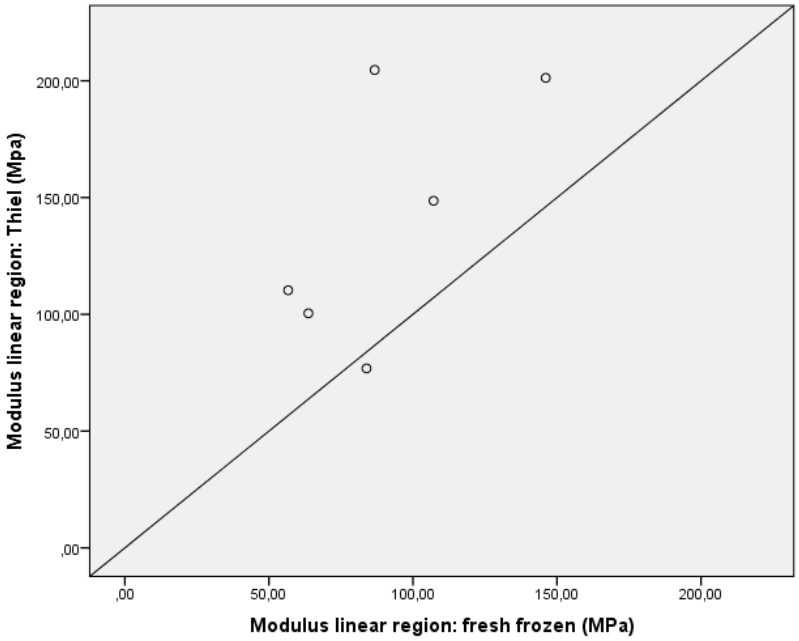


Fig 12: Scatter plot with line of identity. Modulus of linear region of fresh frozen tendons vs. modulus of linear region of Thiel embalmed tendons.

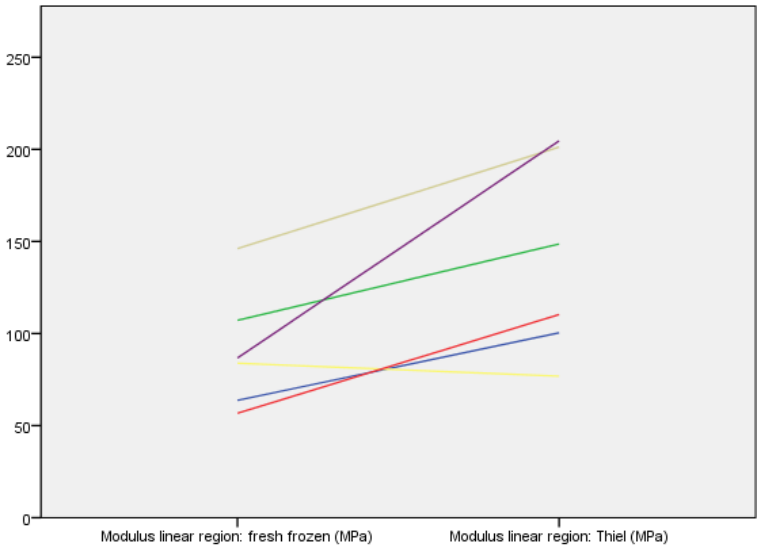


Fig 13: Line chart. Modulus of linear region of fresh frozen tendons vs. modulus of linear region of Thiel embalmed tendons.

4.2 Comparison of biomechanical properties between non-dried and dried embalmed tendons

4.2.1 Fresh tendons

4.2.1.1 Toe

The modulus of the toe-region is not significantly different between the non-dried and dried fresh frozen tendons (P=0,345). The general trend is that dried tendons are stiffer, with a mean difference in modulus of 0,17 MPa ± 17,63 and with a range of 47,55 MPa.

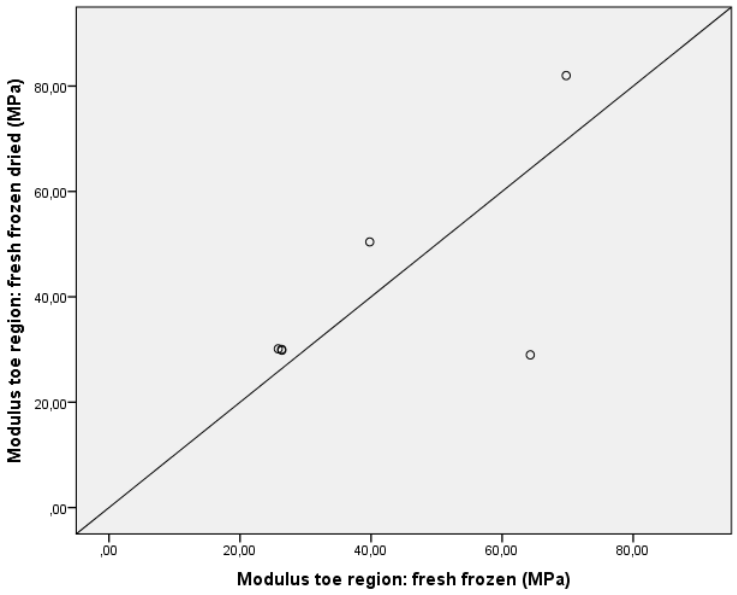


Fig 14: Scatter plot with line of identity. Modulus of toe-region of fresh frozen tendons vs. modulus of toe-region of dried fresh frozen tendons.

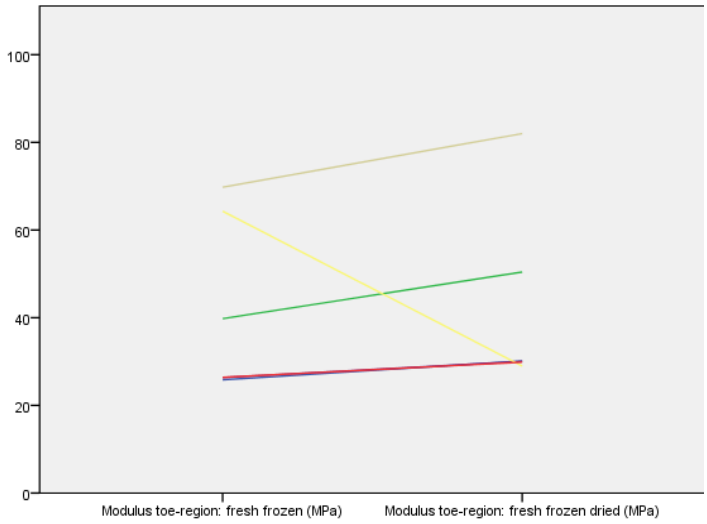


Fig 15: Line chart. Modulus of toe-region of fresh frozen tendons vs. modulus of toe-region of dried fresh frozen tendons.

4.2.1.2 Linear

The modulus of the linear region is significantly different between the non-dried and dried fresh frozen tendons (P=0,046). Dried fresh frozen tendons have a greater modulus than non-dried fresh frozen tendons. The mean difference in modulus was 20,83 MPa ± 17,88 and the range was 52,83 MPa.

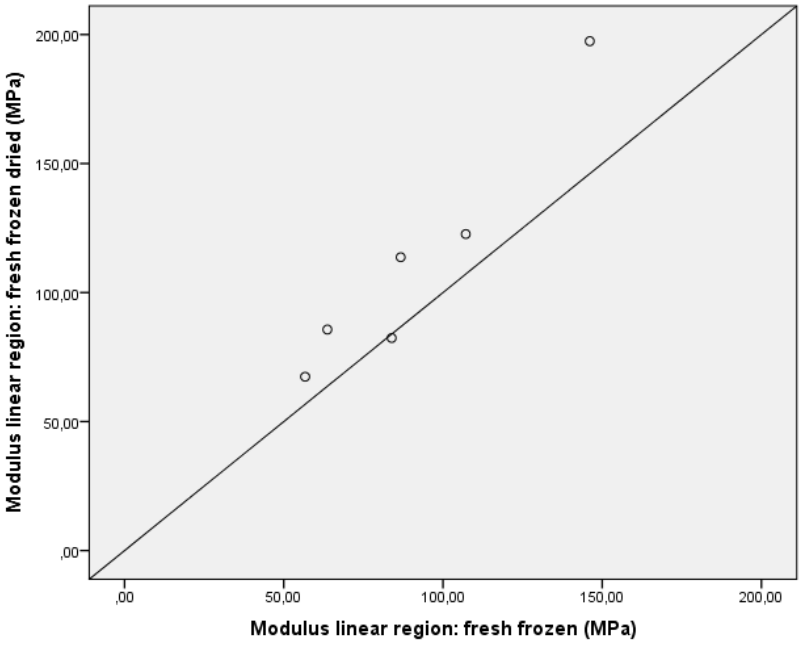


Fig 16: Scatter plot with line of identity. Modulus of linear region of fresh frozen tendons vs. modulus of linear region of dried fresh frozen tendons.

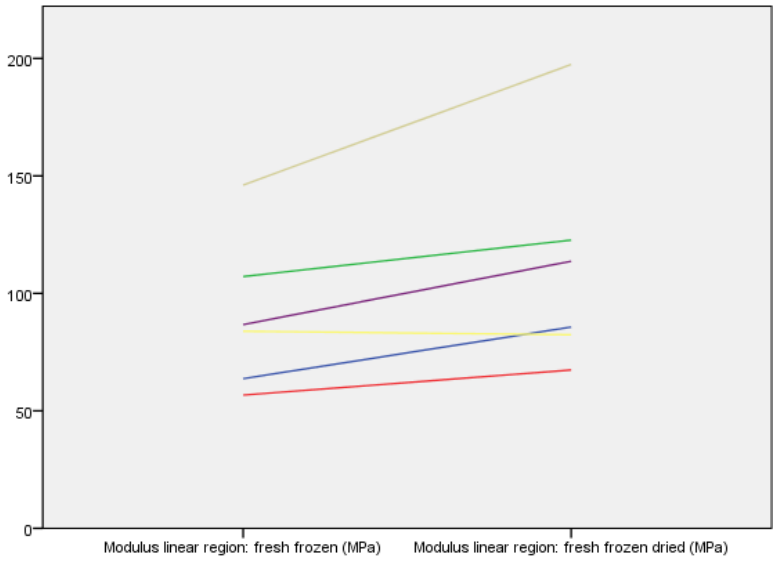


Fig 17: Line chart. Modulus of linear region of fresh frozen tendons vs. modulus of linear region of dried fresh frozen tendons.

4.2.2 Thiel embalmed tendons

4.2.2.1 Toe

The modulus of the toe-region is not significantly different between the non-dried and dried Thiel embalmed tendons (P=0,225). There is no visible general trend. The mean difference in modulus is 18,08MPa ± 36,66 and with a range of 89,40 MPa.

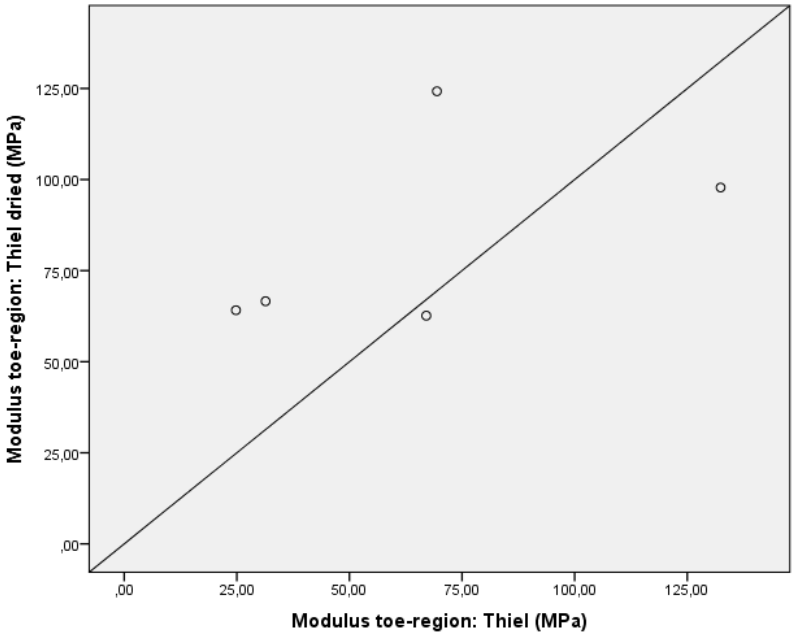


Fig 18: Scatter plot with line of identity. Modulus of toe-region of Thiel embalmed tendons vs. modulus of toe-region of dried Thiel embalmed tendons.

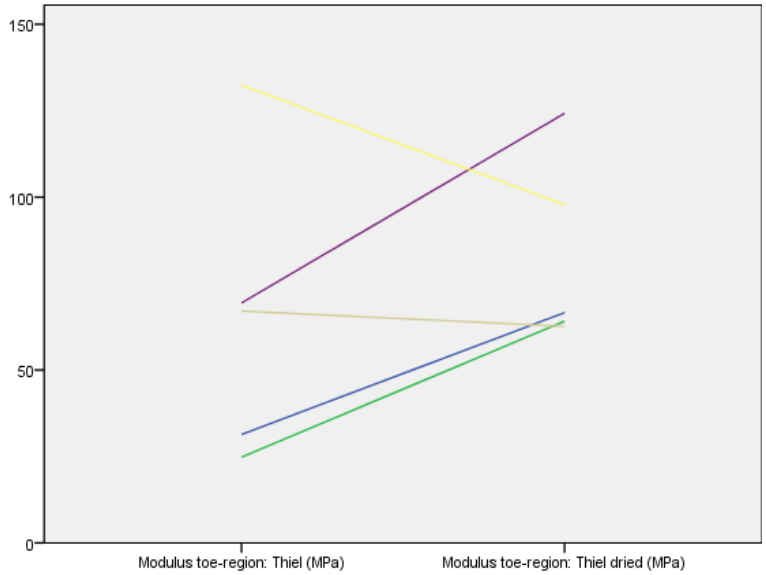


Fig 19: Line chart. Modulus of toe-region of Thiel embalmed tendons vs. modulus of toe-region of dried Thiel embalmed tendons.

4.2.2.2 Linear

The modulus of the linear region is not significantly different between the non-dried and dried Thiel embalmed tendons (P=0,225). There is a general trend towards a greater stiffness of the dried Thiel tendons compared to the non-dried Thiel tendons. The mean difference in modulus is 17,49MPa ± 31,97 with a range of 81,72 MPa.

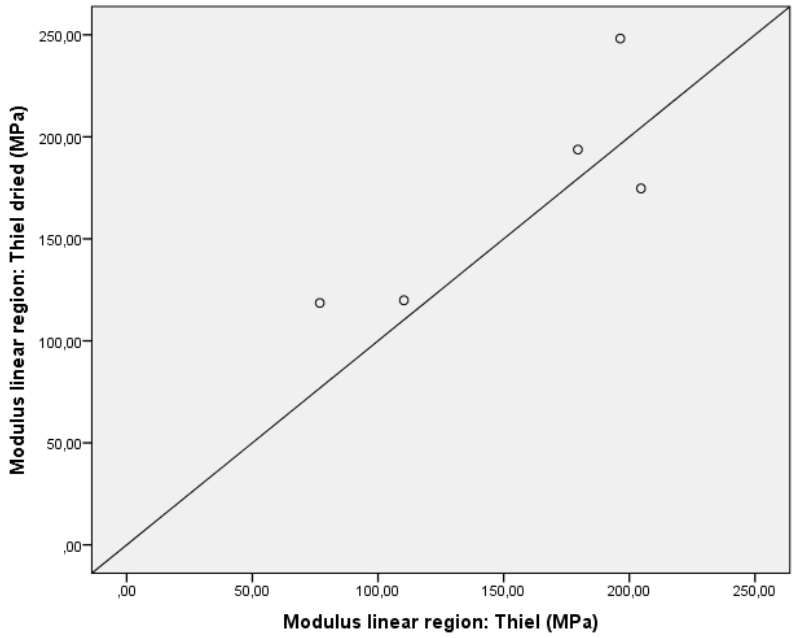


Fig 20: Scatter plot with line of identity. Modulus of linear region of Thiel embalmed tendons vs. modulus of linear region of dried Thiel embalmed tendons.

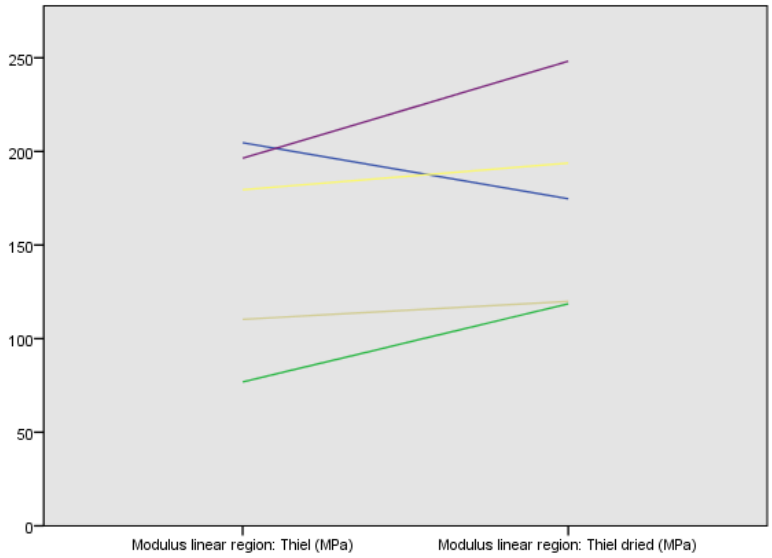


Fig 21: Line chart. Modulus of linear region of Thiel embalmed tendons vs. modulus of linear region of dried Thiel embalmed tendons.

4.3 Overview

4.3.1 Table

Table 1: Overview of the moduli.

		Modulus (MPa)					
		Toe-region		(mean±SD)	Linear region		(mean±SD)
Fresh frozen	non-dried	42,07	±	20,12	90,71	±	32,52
	dried	41,90	±	21,32	111,53	±	46,85
Thiel	non-dried	63,50	±	33,01	152,25	±	50,68
	dried	83,09	±	27,20	171,03	±	54,42

4.3.2 Graphical

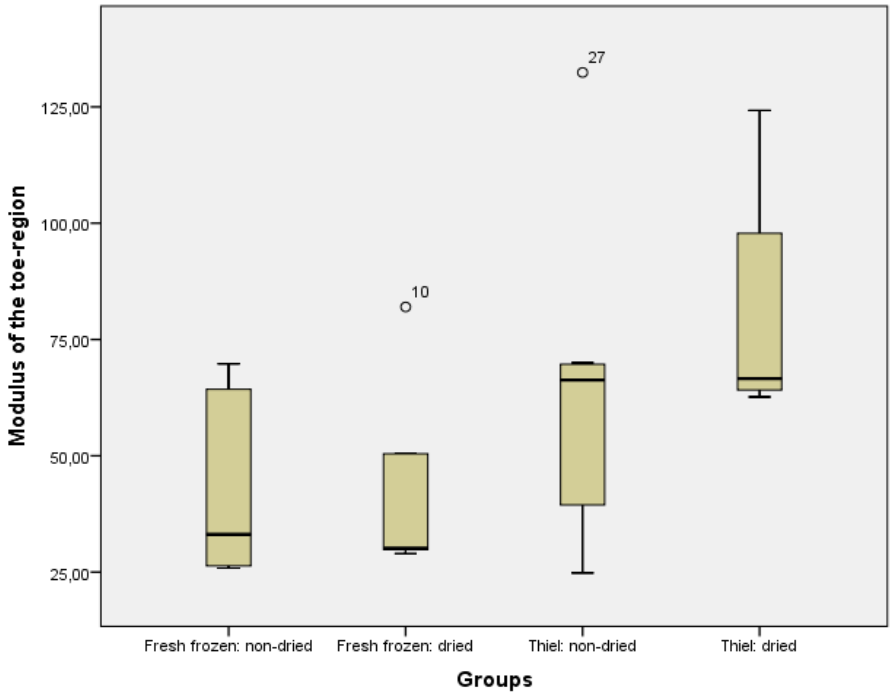


Fig 22: Toe-region: a non- paired comparison of the modulus of the toe-region, represented by a box-plot (P=0,119 (Kruskal Wallis-test))

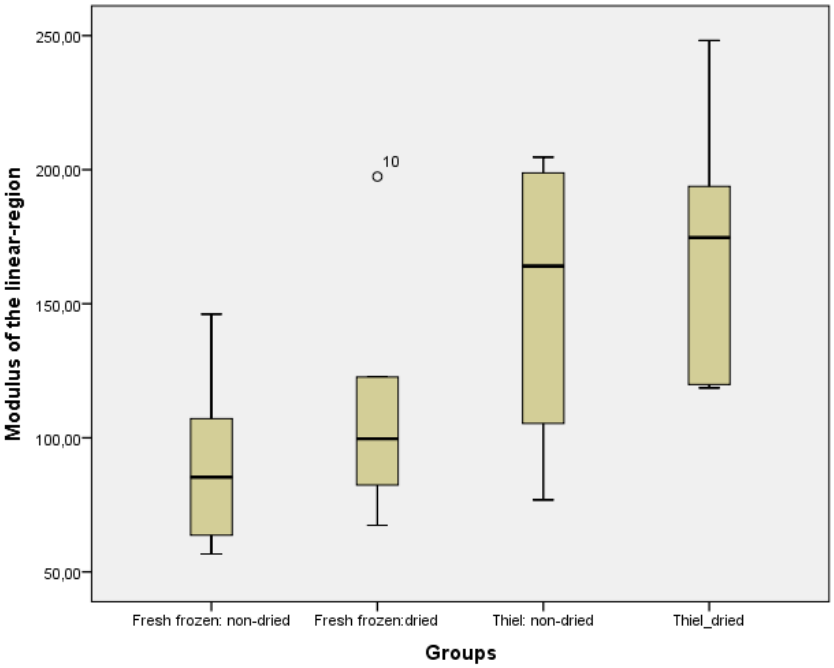


Fig 23: Linear-region: a non- paired comparison of the modulus of the linear region, represented by a box-plot. P=0,046 (Kruskal-Wallis-test)

4.4 Histological analysis

As demonstrated in the images below, a slight modification of the microscopic structure can be seen after Thiel embalming procedure, regardless of the staining procedure. Instead of a straight alignment as in the fresh frozen samples, the alignment is disturbed and rather wavy. In the tendon fibres of the Achilles tendon near the gastrocnemius muscle, the disturbance of the integrity is even greater.

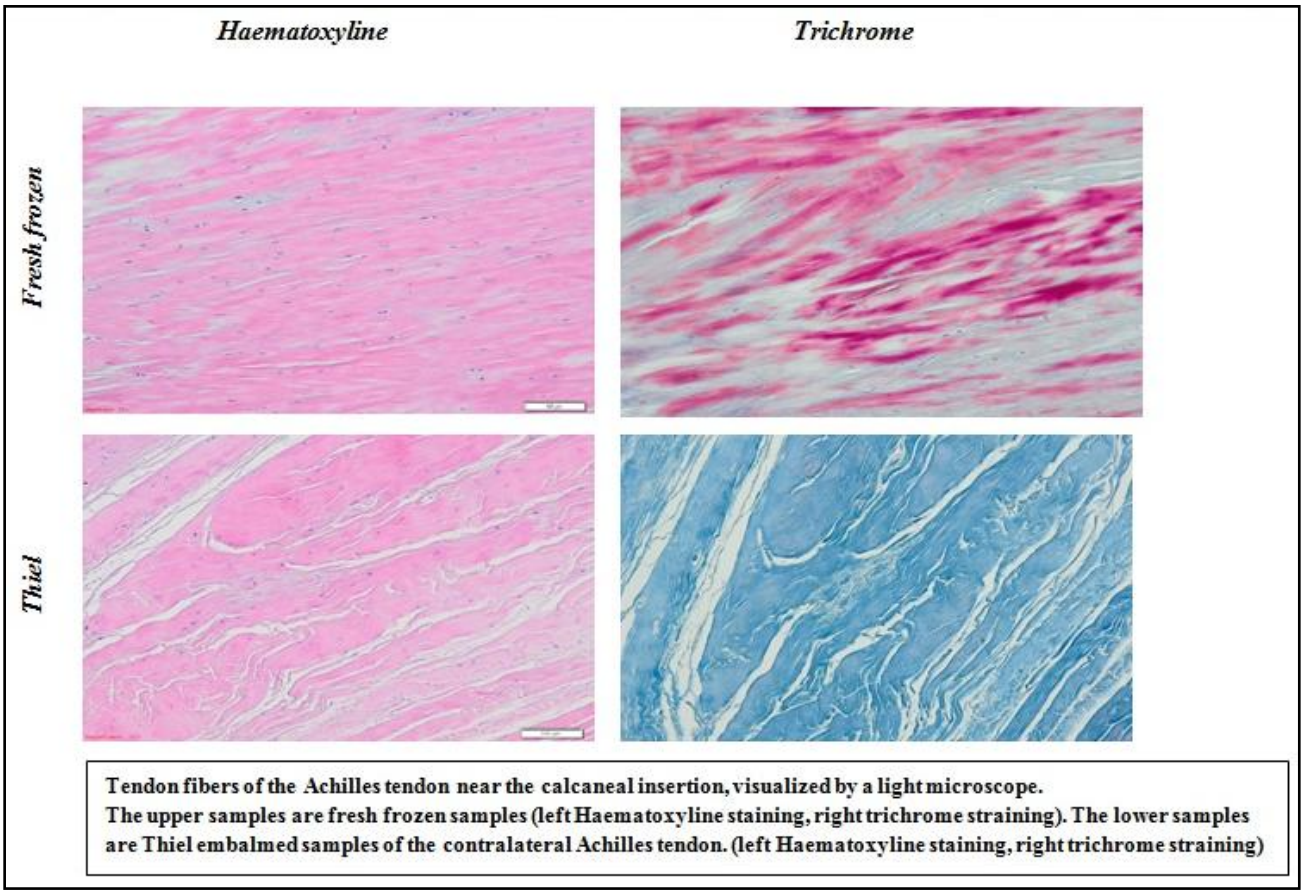


Fig 24: Fibres near the calcaneal insertion.

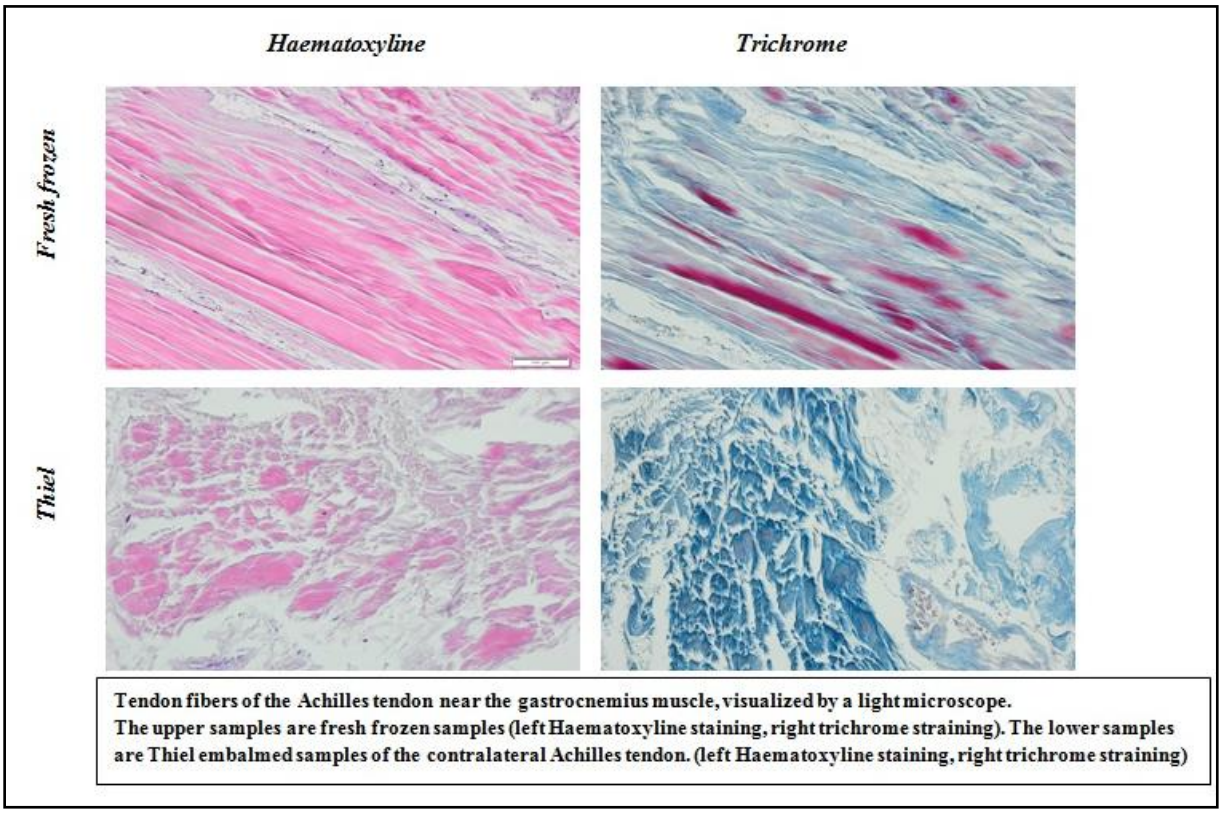


Fig 25: Fibres near the gastrocnemius muscle.

5. Discussion

5.1 Primary objective: Thiel embalming versus fresh frozen

5.1.1 Biomechanics

The main purpose of this study was to investigate whether Thiel embalming of human tendon tissue alters the biomechanical properties in comparison to thawed fresh frozen tendons. Taking into account all the advantages of Thiel embalming found in literature, it would be very convenient to use Thiel embalmed tissue in biomechanical studies.

However, this study demonstrates that Thiel embalming significantly alters biomechanical properties of tendons and is thus not suitable for biomechanical testing. More specifically, the modulus of the linear portion of the stress-strain curve increases significantly as compared to the thawed fresh frozen tendon. The modulus of the toe-region does not change significantly but a trend in increasing modulus after Thiel embalming is clearly observed.

5.1.1.1 Contrast with other studies: Achilles tendons do not soften after Thiel embalming

Our results are in contrast with the results reported in other studies that examined the effect of Thiel embalming on the biomechanical properties of human tissue (9;14;15). These studies observed a trend towards a lower modulus after Thiel embalming. The study of Fessel et al. which also examined the effect on tendon tissue (flexor digitorum profundus tendon and fascicles of rat tail tendons), concluded that the elastic modulus tends to decrease after Thiel embalming (14). Two hypotheses can be posed to explain this discrepancy between the results of our study and the study of Fessel et al.:

1. Difference in testing protocol: the tests of Fessel et al. on human tendon specimens had numerous limitations. The number of tested specimens was limited to three cadavers, the embalming time was uncontrolled and human specimens were not controlled for age, gender, lifestyle and other donor factors. Previous studies illustrated that biomechanical properties are strongly dependent of donor factors such as race, age and other variables (28;29). Consequently, these elements may strongly bias the results. The current study overcomes these limitations by a greater number of tested specimen (7 cadavers), a fixed embalming time of 6 weeks and a paired comparison of the right and left tendon of the same person.

2. Difference in tissue perfusion kinetics between different kind of tissue: As mentioned above, Fessel et al. also examined the effect of Thiel embalming on rat tail tendon fascicles (RTTF's). This testing protocol was more robust and also demonstrated a lower modulus after Thiel embalming (P<0,05) (14).

   The authors stated that the degree of tissue softening by Thiel embalming is highly dependent on time of exposure to the Thiel solution (and mainly the denaturation influence of the boric acid in it) and that different tissue perfusion kinetics of Thiel solution could explain some of the variability within and between tissues extracted from human cadavers (14). So another explanation why the thin RTTF's and FDP tendons 'soften' and Achilles tendons do not, could be that in Achilles tendon diffusion is more limited by its thickness.

   This could also form an explanation for the discrepancy between the results of our study and results of research which examined the effect of Thiel embalming on the biomechanical properties of non-tendon tissue. Wilke et al. for example concluded that flexibility parameters of spinal motions in specimens increased with the Thiel embalming process (9) and Unger et al. showed that six month of Thiel storage resulted in a reduction of elastic modulus as well as an altered failure mode of bone specimens (15).

5.1.1.2 Increase of modulus of Achilles tendons after Thiel embalming

Nonetheless, the reason why stiffness of Achilles tendons even increases, remains hereby unexplained. The studies mentioned above state that Thiel embalming has an opposite effect on tissue biomechanics compared to other classical (formalin based) preservation methods which cause stiffening of the tissue (5;7;9;14;15)**.** In the current study however, it is demonstrated that the effect of Thiel embalming on the biomechanical properties of Achilles tendons is similar to the effect of classical embalming techniques, namely an increase of the modulus.

The explanation for the effect of classical embalming techniques on biomechanical properties has already been identified: Aldehydes (like gluteraldehyde, formaldehyde) in high concentration cause extensive cross-linking in collagen (linking of collagen lysine and hydroxylysine side chains). Hereby, the overall collagen structure is stabilized and the slippage between neighbouring collagen molecules is reduced, which implies additional strength and stiffness of the tissue (7). In Thiel embalming fluid, the concentration of aldehydes is very low. Therefore, it is not clear whether this process of cross-linking also takes place in Thiel embalmed bodies. Furthermore, the possible collagen cross-linking due to

the formalin, is opposed by the denaturing effect of the boric acid in the Thiel solution (14) (Boric acid is suggested as the cause of observed denaturation and softening of tissue, as boric acid is the only acid in Thiel's mixture and acids are well-known to have corrosive effects on proteins (16)).

However, the hypothesis that the effect of Thiel embalming varies within and between tissue because of different tissue perfusion kinetics of (substances in) Thiel solution (14), could also form an explanation. It might be that in the relatively thick Achilles tendon, the formaline diffuses more easily than the boric acid (which is less water-soluble and consists of larger molecules). This way, Achilles tendons are more affected by collagen cross-linking due to the (low concentration of) formaldehyde and less affected by the denaturizing effect of Boric acid. Furthermore, Hansen et al. stated that subtle ultrastructural differences between various collagen tissues could evoke very different mechanical effects of cross-linking (7), which fits well within this hypothesis.

Further research should include an atomic force microscopy (AFM) investigation to demonstrate that Thiel embalming (with its low concentration of formaline) also causes a sufficient amount of crosslinking to explain the higher modulus found in Achilles tendon after Thiel embalming.

In conclusion, the results of our study, in addition to the observation of muscular changes induced by Thiel (17;16) and the results of the other studies which examined the biomechanical properties of Thiel embalmed tissue (9;14;15)¸ confirm that Thiel embalmed tissue is not suited for studying the in vivo biomechanical properties of human tissue.

5.1.2 Histology

In the current study, also a histological analysis was performed to search for an explanation for the changes in biomechanical properties. After Thiel embalming, a slight modification of Achilles tendon fibres could be seen: the alignment becomes relatively wavy. This might suggest that collagen of tendon fibres are indeed slightly affected by the corrosive effect of boric acid.

This result is not entirely similar to the findings of Benkhadra et al., who examined the histological effect of Thiel embalming on biceps brachia muscle and the tendon of the brachioradialis muscle. According to their report, collagen fibres in muscle and tendon remained undisturbed after Thiel embalming (in contrast with the observed damage of muscle

fibres in their study, which could form an explanation for the overall exceptional suppleness of Thiel embalmed cadavers as described in most studies) (16).

The result of Benkhadra et al. and our own results, which only indicate a minor modification, correspond with our biomechanical findings that Achilles tendons do not predominantly denaturize and soften.

The possibility of alterations in the collagen ultrastructure (e.g. crosslinking) however cannot be excluded with an optical microscope and, as mentioned above, an atomic force microscopy study should be performed.

5.2 Secondary objective: exposure to room conditions

Our secondary objective was to find out to whether biomechanical properties of both thawed fresh frozen tendons and Thiel embalmed tendons are influenced by exposure to room conditions (room temperature, room humidity,..) and the resultant dehydration and degeneration. As mentioned earlier, in most studies a lot of attention is paid to preclude dehydration of human tissue in studies examining its biomechanical properties. However, to our knowledge, there are very few studies which quantified the effect of dehydration on the biomechanical properties of human tissue (32;33). To examine the effect of dehydration, we exposed the clamped Achilles tendon between 2h30 and 3h to room conditions after the first test (see materials and methods). We considered this as a realistic period of time in which moistening of the tissue is neglected in some studies.

Experience with the handling of dehydrated tendon and especially molecular analysis suggests that drying alters the biomechanical properties of tendon and other tissue. The main component of tendon is protein (collagen) and as a polar molecule, water interacts to a great extent with hydrophobic and hydrophilic elements of the proteins (30;31). The dehydration process causes the breakage of the hydrogen bonds, leading ultimately to the loss of the collagen triple helix structure. As the biomechanical properties of tissue greatly depend on the molecular structure, it is obvious that the extraction of moist changes the biomechanical properties (32). However, during the embalming procedure, a great part of the water in the body (also in the tendons) is replaced by other fluids. The mechanisms that take place in embalmed tendons when exposed to room conditions are consequently not entirely clear.

Our results demonstrated that exposure of thawed fresh frozen human tissue to room conditions without moistening, significantly alters the biomechanical properties, more

specifically the modulus increases. It can thus be stated that in studies where moistening of tissue is impossible for longer than two hours, results should be interpreted with caution. An additional study would be opportune to examine the duration of non-moistening after which the biomechanical properties change significantly.

In the current study, a significant alteration of biomechanical properties of Thiel embalmed tendons when exposed to room conditions could not be demonstrated. However, a trend towards an increased modulus was seen. When biomechanical tests are performed on Thiel embalmed tissue (keeping in mind the limitations of testing on Thiel embalmed tissue, as described above), keeping the tissue well moistened is slightly less important. This could be an advantage in studies where keeping the tissue well moistened is not always possible.

5.3 Toe-region

In the context of the first and second objective, we also took a closer look at the toe-region of our specimens and their mutual differences. Although this toe-region is often considered as unimportant, characterization of the toe-region is indispensable to assess the biomechanical properties of tissue and even has practical value. Arguments to prove that the toe-region is a distinct identity which one ought to pay attention to, are for instance:

- In the activities of daily living some structures (e.g patellar grafts as replacement for a ruptured ACL) are only subjected to relatively small amounts of stress and strain and these low strains will probably elicit a response that corresponds with the toe-region and not the linear region (17).
- A subject-to-subject variability is found in the toe-region properties of patellar tendon, which can partially be explained by donor factors, similarly to the linear region.

In this study a modulus of the toe-region and a transition point from toe-region to the linear region was calculated through a bilinear fit model. The toe-region is approximated as a straight line even though the toe-region is non-linear. However Naveen et al. confirmed that a linear approximation provides a conservative estimate for the onset of the transition from the toe-region to the linear region (17) (A discussion about the transition point from toe-region to the linear region and the values of the toe moduli can be found in ‘addendum 3’).

If the toe-region of the tendons does not differ between fresh frozen and Thiel embalmed tendons, it could be stated that in studies examining biomechanical properties of the toe-region area, Thiel embalmed tendons can be used as well. Indeed, in the current study no

significant difference in modulus of the toe-region or difference in transition point from toe-region to linear region was found between fresh frozen and Thiel embalmed tissue. However, there is a trend that Thiel embalmed tendons have a higher modulus of the toe-region than fresh frozen tendons. A greater number of tendons, should be investigated to examine whether a significant difference can be found. Consequently, it is not certain whether toe-region of Thiel embalmed tendon can be considered as representative for the in-vivo state of the tendon's toe-region.

Furthermore, dehydration of both fresh frozen and Thiel embalmed tendons seems to increase the modulus of the toe-region in a non-significant way.

5.5 Notes and limitations concerning the testing protocol

Several notes can be made about the current study whichare broadly described in addendum 4. They are briefly listed here:

- Slippage:

  Clamping methods as used in this study are known to have a certain amount of slippage out of the grips when heavily loaded (1). Therefore, we quantified the slippage out of the grips of each tendon, to verify that our results were not falsified by slippage. This was confirmed, as slippage never differed significantly between fresh frozen, Thiel embalmed and dried tendons.

- Repetitive loading:

  In our study we examined the effect of tendon dehydration on the biomechanical properties of the fresh en Thiel embalmed Achilles tendon. Each tendon was therefore tested twice (the second time after 2-3 hours of exposure to room conditions). A remark that could be made is that the alterations of biomechanical properties of the tendon in this setting might also be due to repeatedly loading instead of the drying out. However, data from literature indicates that the amount of load used in the current study will not significantly alter biomechanical properties.

- Preloading:

  When the same tendon is tested more than once, preloading is necessary to obtain reproducible data. Haraldsson et al. proved that data were achieved already after one cycle of preconditioning to a stress of 3–4 Mpa (1). This amount of stress corresponds well to the preloading stress in our study. Consequently alterations of biomechanical properties in different conditions of the tendon are considered to be the result of the

dehydration process itself and not related to unreliable or non-reproducible results of the tests.

- Loading:

  To obtain a more complete representation of the biomechanical properties, also yield stress and strain values and failure stress and strain values should be examined. Because of the fact that in this study the tendons needed to be examined twice (before and after dehydration), the specimens were not tested till failure.

- Measuring methods:

  DIC strain measuring method was used on four Achilles tendons. Therefore, these tendons are stained with methylene blue. It is not yet known whether this staining has an influence on the biomechanical properties.

6. Conclusion

This study demonstrates that Thiel embalming significantly alters the biomechanical properties of tendons and is thus not suitable for biomechanical testing. The results of this study, demonstrating an increase of the modulus of the linear portion of the stress-strain curve after Thiel embalming, are in contrast with other studies mentioning a decrease of the modulus of several tissues after Thiel embalming (9;14;15).

Concerning the effect of dehydration on biomechanical properties on human tissue, our results demonstrated that exposure of thawed fresh frozen human tissue to room conditions without moistening for two hours, significantly alters the biomechanical properties, more specifically by increasing the modulus. Consequently, it can be stated that in studies where moistening of tissue is impossible for longer than two hours, results should be interpreted with a lot of caution. A significant alteration of biomechanical properties of Thiel embalmed tendons when exposed to room conditions and possible dehydrations could not be demonstrated.

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8. Addenda

7.1 Addendum 1:

7.1.1 Embedding in paraffin

- Tissue fixation in neutrally buffered formalin 4%
- Alcohol 30% 1 hour
- Alcohol 50% 1 hour
- Alcohol 70% 1 hour
- Denaturated alcohol (alcohol 96%) 1 hour
- Isopropylalcohol 1 hour
- Isopropylalcohol / toluol(ratio 1/1) 1 hour
- Toluol 30 min.
- Paraffin at 60°C

7.1.2 Haematoxylin-eosin-staining

Bath 1	toluene	5’
Bath 2	toluene	5’
Bath 3	toluene	5’
Bath 4	isopropanol	2’
Bath 5	isopropanol	2’
Bath 6	alcohol 96%	2’
Bath 7	alcohol 96%	2’
Bath 29	aqua	2’
Bath 19	aqua destillated	1’
Bath15	haematoxylin	15”
Bath 29	aqua	2’
Bath 16	clarifier I	1’
Bath 29	aqua	1’
Bath 17	bluing reagent	1’
Bath 29	aqua	1’
Bath 19	aqua destillated	1’
Bath 18	eosin+phloxine	30”
Bath 29	aqua	2’
Bath 20	alcohol 96%	2’
Bath 21	alcohol 96%	2’
Bath 22	isopropanol	2’
Bath 8	isopropanol	2’
Bath 9	toluene	2’
Bath 23	toluene	2’
Bath 38	toluene	1’

7.2 Addendum 2

7.2.1 Composition of Thiel embalming fluids

IMMERGING SOLUTION	
Water	720 L.
Ethylene glycol	71,9 L.
Formalin	14,4 L.
Stock solution B	14,4 L.
Boric Acid	21,6 Kg.
Ammonium nitrate	71,9 Kg.
Potassiumnitrate	36 Kg.
SodiumSulfite	50 Kg.
TOTAL	1000,2 L.

STOCK SOLUTION A	
Water	**63,3 L.**
Ethylene glycol	**19 L.**
Boric acid	**1,9 Kg.**
Ammonium nitrate	**12,6 Kg.**
Potassiumnitrate	**3,2 Kg.**
TOTAL	**100,00 L.**

STOCK SOLUTION B	
Ethylene glycol	**18,2 L.**
4-chloro-3-methyl-phenol	**1,8 Kg.**
TOTAL	**20 L.**

Perfusion fluid for 1 cadaver

Cadaver Weight -80 Kg		Cadaver Weight + 80Kg	
Stock solution A	**14,3 L.**	Stock solution A	**19 L.**
Stock solution B	**0,5 L.**	Stock solution B	**0,6 L.**
Formaline	**0,3 L.**	Formaline	**0,4 L.**
Sodiumsulfite	**0,7 Kg.**	Sodiumsulfite	**0,9 Kg.**
TOTAL	**15,8 L.**	**TOTAL**	**20,9 L.**

7.2.2 Comparison of the Thiel embalming fluid used in this study and the study of Fessel et al.

Solution A: Almost identical.

solution B: In the study of Fessel et al, solution B contains ethylene glycol 10% (V/V), and 4-chloro-3-methylphenol 1% (V/V). In this study the concentration of ethylene glycol in solution B is about 90% (V/V) and about 10% 4-chloro-3-methylphenol.

Immerging solution: Identical except for composition of solution B.

Bath solution: Almost identical. The amount of formaline is even slightly smaller in the bath solution used in this study. (1.44% (V/V)) vs 2% (V/V)).

7.3 Addendum 3: Transition point

As mentioned earlier, it is possible with the bilinear fit model to calculate the transition point (e*) between the toe-region and the linear region. Like the modulus of the toe-region, also the transition point never changes significantly in this study (see table 2 and 3).

Chandrashekar et al. also used the bilinear fit model for calculating the modulus of the toe- and linear region of patellar tendons and the transition point between the two (35). The findings of this study show that, based on a bi-linear approximation, the strain at which the patellar tendon transits from the toe-region to its linear region (e*) is about 5%. This is consistent with our results where the transition point was slightly less than 5% in the fresh frozen group and 3% in the Thiel embalmed group. In our study, the mean modulus of the toe-region was about 41,97 MPa in the fresh frozen group and 73,30 MPa in the Thiel embalmed group. The modulus of this study is remarkably lower than those found by Chandrashekar et al. They found a mean modulus of 122 MPa. The mean linear modulus found in this study was 101,12 MPa in the fresh frozen group and 161,64 MPa in the Thiel embalmed group. This is remarkably lower than the value found by Chandrashekar et al. They found a mean value of 485MPa.

Table 2: It is possible with bilinear fit (1) to calculate the transition point (ε*) between the toe-region and the linear region. (see table). Like the modulus of the toe-region, also the transition point never changes significantly. The mean of the transition points ranged from 3% to 5%.

		E* (mean ± SD)
Fresh frozen	non-dried	0,05 ± 0,02
	dried	0,04 ± 0,01
Thiel	non-dried	0,03 ± 0,01
	dried	0,03 ± 0,01

Table 3: Transition point (Wilcoxon signed ranks test).

Transition point (Wilcoxon signed ranks test)	P
Fresh frozen versus Thiel	0,273
Fresh frozen non-dried versus dried	0,068
Thiel non-dried versus dried	0,564

7.4 Addendum 4: Notes concerning the testing protocol

7.4.1 Slippage

In this study, grip-to-grip moduli ranged from 56,70MPa to 248,18 MPa. These values are much lower than the moduli calculated in the study of Wren et al. who found moduli that ranged from 500 to 1850 MPa (36). This difference in value of the modulus might be explained by slippage out of the grips.

Indeed, clamping methods as used in this study are known to have a certain amount of slippage out of the grips when heavily loaded (1) . Therefore we quantified the slippage out of the grips of each tendon, to verify that our results were not falsified by slippage. This was confirmed, as slippage never differed significantly between fresh frozen, Thiel embalmed and dried tendons. (see tables below)

Table 4: mean slippage in each condition, measured with the DVRT's

DVRT-measuredslippage	Slippage (mean±SD)
Freshfrozen: non-dried	1,6% ±1,2
Freshfrozen: dried	0,6% ± 0,5
Thiel: non-dried	2,20%± 0,8
Thiel: dried	1,13%± 2,68

Table 5: P-values of the Wilcoxon signed rank test. With this test DVRT measured slippage was compared between the tested groups. No significant difference in slippage can be demonstrated

Wilcoxonsigned rank test	P
Non-dried freshfrozen versus non-dried Thiel	0,481
Non-dried freshfrozen versus dried freshfrozen	0,059
Non-dried Thiel versus dried Thiel	0,705

Table 6: X-values (see M&M) of the tendons on which the DIC measuring method was executed . The X-value ranged from 12% to 51%

DIC		Tendon 1 (X-Value)	Tendon 2 (X-value)
Freshfrozen:	non-dried	43 %	51 %
	dried	39 %	43 %
Thiel:	non-dried	12 %	36 %
	dried	21 %	28 %

Table 7: P-values of the Wilcoxon signed rank test. With this test the X-values were compared between the tested groups. No significant difference in X-values can be demonstrated

Wilcoxonsigned rank test	P
Non-dried freshfrozen versus non-dried Thiel	0,180
Non-dried freshfrozen versus dried freshfrozen	0,180
Non-dried Thiel versus dried Thiel	0,655

However the comparison of DIC quantified slip reveals that Thiel embalmed tendons seem to have smaller X-values (not statistical significant). This indicates that the slippage is slightly greater in those tendons as compared to fresh frozen tendons. This trend is also slightly seen with the DVRT slippage-measurement technique (also not significant) and might be due to the fact that the tendon is more slippery because of Thiel fluid. This finding indicates that the modulus calculated for Thiel embalmed tendons may be underestimated and that the difference in modulus between fresh frozen and Thiel embalmed tendons may be even larger than the significant difference we have already found.

Besides, a trend of reduced slippage can be seen in the dried tendons as compared to the non-dried tendons with the DVRT- measured slippage (not statistical significant). Effect of increase of modulus by dehydration might therefore be exaggerated.

The lowest X-value in this study was 12% ± 1 (achieved in a Thiel embalmed tendon), which indicates a major slippage. It is therefore important that the slippage is maximally reduced in further studies. A solution could be the use of freeze clamps (36). Another solution could be midsubstance measurement instead of grip-to-grip measurement. It is well known that calculations based on grip-to-grip measurement typically give a higher strain value and subsequent a lower modulus value than those based on midsubstance measurements(1).

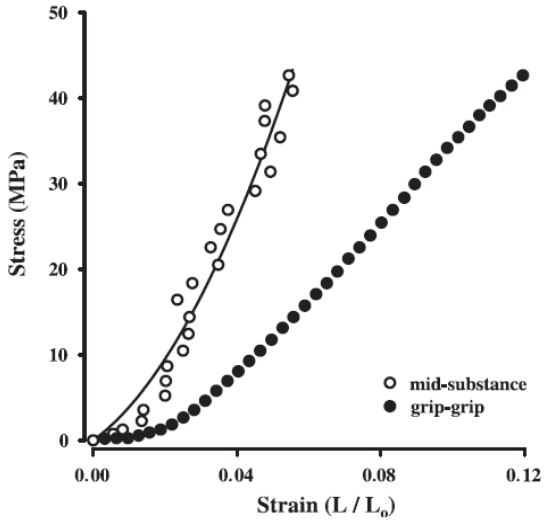

Fig 26: Graph illustrating the difference in stress-strain curve of a specimen when strain was measured midsubstance or calculated by grip-to-grip measurement. (1)

Table 8: The calculation of strain based on grip-to-grip deformation and tendon midsubstance deformation based on optical markers (1).

Reference	Tendon Tissue	Grip-Grip Strain, %	Midsubstance Strain, %
Wren et al. (37)	Human Achilles tendon	12.8	7.5
Butler et al. (6)	Human patellar tendon	30.2	12.0
Dressler et al. (10)	Rabbit patellar tendon	15.5	7.1
Devkota et al. (9)	Avian flexor tendon	16.4	12.2
Wu et al. (38)	Rat tibialis anterior tendon	15.8	3.7
Present study	Rat tail fascicle	4.3	2.6
Present study	Human patellar fascicle, anterior	12.4	6.8
Present study	Human patellar fascicle, posterior	13.0	8.7

Note that, in all cases, grip-grip strain exceeds that of midsubstance strain.

The table above illustrates that the strain value differs between grip-to-grip and midsubstance strain measurements and consequently the modulus value. The reason for the increase in modulus in the mid-substance group is due to absence of influence of slippage in this measurement technique. Midsubstance measurement of the modulus can be achieved by DIC, which has the additional advantage that local strain can be measured in different directions (1). DVRT's attached directly to the tendon are an alternative, but this induces a risk of disturbing the anatomical integrity of the tendon, which could result in a change of the biomechanical properties.

7.4.2 Repetitive loading

In our study we examined the effect of tendon dehydration on the biomechanical properties of the fresh en Thiel embalmed Achilles tendon. Each tendon was therefore tested twice: the second time after 2-3 hours of exposure to room conditions. A remark that could be made is that the alterations of biomechanical properties of the tendon in this setting might also be due to repeatedly loading instead of the drying out.

In our setting we maximally loaded the tendon to 80kg, which is equivalent to a mean stress on the tendons of 10,10 Mpa and in the smallest tendon this resulted in a stress of 14,82 Mpa. The literature concerning the influence of repeatedly loading on tendon confirms that this amount of stress and strain does not influence biomechanical properties (37). Chandrasekhar et al for example concluded in their study that cyclic loading of the human patellar tendon grafts up to 33% of their failure stress will not change the biomechanical properties of the graft. Extrapolating these data of patellar tendons to our samples, this would mean that any load of less than about 23,43 Mpa (33% of 71 Mpa) does not influence the stiffness of the Achilles tendon (Wren et al found in their study that failure stress of Achilles tendons is about 71 MPa ± 17(36)). The maximum stress seen (in the smallest tendon) was 14,82 Mpa and this is well below the limit value of 23,43Mpa. Also Devkota et al (38) concluded that loading tendons to low levels of stress and strain doesn't influence the biomechanical properties. They demonstrated that no change in strength and stiffness properties of patellar tendon could be observed with ramp loading to strain limits ranging from 1% to 14%. In our study the maximum strain observed was 12%. Consequently, We can conclude that alteration in biomechanical properties is caused by the dehydration process itself and not due to repeated loading.

7.4.3 Preloading

Before each test, tendons were preloaded in this study with a load of 350N for two minutes (this is equivalent to a mean preloading strain of 6±1 % and a mean preloading stress of 4,54 MPa ± 1,34. The maximum preload stress (in the smallest tendon) was 7,23 MPa). Preloading causes a rightward shift of the stress-strain curve and is therefore required to obtain reproducible data (1). Based on results from the rat tail fascicles, Haraldsson et al proved that reproducible data were achieved already after one cycle of preconditioning to a stress of 3–4 Mpa (1). This amount of stress corresponds well to the preloading stress in our study. Consequently, alterations of biomechanical properties in different conditions of the tendon are not related to unreliable or non-reproducible results of the tests.