The Fracture Stress of Rat Achilles Tendons

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Summary

For the determination of the fracture stress of soft tissues both the fracture force and the cross sectional area are required. For short tissues these prerequisites are difficult experimental tasks. The determination of the fracture force necessitates proper gripping without damaging the tissues or altering their properties. In order to meet this challenge the rapid-freezing technique was employed, modified to ensure that the tendon was not frozen. On the other hand an accurate value of the cross sectional area of short soft tissues is difficult to be obtained using conventional techniques. In this context a novel procedure is proposed here based on the histologically-measured cross-sectional area of the dehydrated tendon after the biomechanical testing. Combination of these solutions permitted the performance of tension tests for rat Achilles tendons and calculation of their fracture stress. The values of the Achilles tendon failure stress, as estimated above, exhibited considerably lower scattering compared to those of the fracture forces.

Introduction

Understanding the biomechanical behavior of the connective tissue is crucial in several fields of medicine, such as Orthopedics. Intensive research is carried out through experimental protocols involving laboratory animals and in particular rodents. The study of tendon biomechanics in small-size laboratory animals is characterized as a highly demanding procedure exhibiting considerable technical difficulties. There are two main technical challenges that have to be met. The first one is the problem of rigidly gripping the tendon without damaging it or altering its mechanical behaviour. Without this basic requirement it is impossible to conduct an accurate and reliable experiment. The second challenge is the reduction of the forces and the displacements imposed to stresses and strains, respectively. The advantage of this reduction is that the latter quantities do not depend on the morphometry of the specific specimens but they rather characterize the material of the tendon itself, eliminating thus the interspecimen variability of the results.

For the problem of rigidly gripping a tendon at high loads without damaging it, several methodologies have been proposed over the last years. The most widely used ones are: a) conventionally gripping using compression and b) clamping the tendon using rapid freezing. The compression of the specimen (using for example serrated grips) alters the biomechanical characteristics of the examined tissue (*Riemersa & Schamhardt*, 1982). In addition slippage is not always avoided even in cases of relatively long tendons (*Cheung & Zhang*, 2006). Especially in the case of very short tendons (like the Achilles tendon of rats) the above mentioned difficulties are intensified rendering the method practically inapplicable. On the other hand the rapid freezing technique results in a more efficient gripping of the tissue with

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reduced compression of the specimen (*Riemersa & Schamhardt*, 1982; *Sharkey et al.*, 1995; *Wieloch et al.*, 2004). This technique was introduced by Riemersa & Schamhardt in 1982 and was further developed by many others. According to them the clamped part of the tendon and the metal clamp are rapidly frozen using liquid CO_2. Rapid freezing is widely used today with relatively good results. The greatest disadvantage of rapid freezing emanates from the technical difficulty to accurately restrict freezing in a pre-defined portion of the tissue. This difficulty is even greater in the case of short tissues such as the Achilles tendons of rats. Since the freezing of the tissue causes significant alterations to its mechanical properties, a failure to control the freezing can undermine the reliability of the study. In order to overcome the above mentioned difficulty, an alternative clamping technique, using rapid freezing, is described in the present paper for the experimental study of the mechanical behaviour of bone-Achilles tendon-muscle units of rats.

Concerning the problem of reducing the forces measured to reliable stress estimations, the different procedures found in the literature could be separated into three main categories according to the time and frequency of the cross-sectional area measurements. Some researchers measure the cross-sectional area before the biomechanical test, either using a calliper (*Nakagaki et al.*, 2007; *Teramoto & Luo*, 2008) or other custom-made devices (*Butler et al.*, 1984; *Loitz et al.*, 1989; *Wu et al.*, 2004). Furthermore others take several real-time measurements of the cross-sectional area of the tendon during the loading using indenter probes connected to high resolution displacement transducers (*Derwin et al.*, 1994; *Huang et al.*, 2004; *Soslowsky et al.*, 1994; *Soslowsky et al.*, 2000). Finally some researchers estimate the cross-sectional area after the end of the test, based on the percentage of collagen in the tendon (*Oxlund et al.*, 1984). For the purposes of the present study a novel post-fracture indirect method for the estimation of the fracture stress of rat Achilles tendons was followed, according to which the cross-sectional area of the dehydrated tendon is identified as an "effective" cross-sectional area (at least for tensile loads).

Materials and Methods

Animals

Male Wistar rats, aged 4 months, were studied, coming from the same breeder (National Research Center of Natural Sciences "Dimokritos"). The animals were supplied by van in filter boxes and quarantined for 2 weeks in the Central Animal Laboratory of the Department of Experimental Surgery and Surgical Research of the University of Athens. The experimental protocol was carried out according to Greek legislation regarding ethical and experimental procedures (Presidential Decree 160/1991, in compliance to the EEC Directive 86/609, and Law 2015/1992, and in conformance with the European Convention "for the protection of vertebrate animals used for experimental or other scientific purposes, 123/1986").

Husbandry

The animals were housed, in an open system, two per cage, in polycarbonate cages of dimensions: 480 x 265 x 210 mm, floor area: 940 cm^2 (2154F, Tecniplast, Italy). Wooden, dust-free, litter was used for bedding, with no pretreatment (Scobis-Uno, Italy). The conditions in the animal house were 15 air changes/hour, with regulated environmental temperature at 22±2 ºC, regulated relative humidity 55±10% and artificial light/dark at 06.00/18.00, using fluorescent lighting ca. 300 lux. All animals were acclimatized to the laboratory conditions for one week period prior to the experiment. The animals were fed *ad libitum* a commercial pelleted food (510K, Greek Animal Food Industry, Greece), with no pretreatment, the nutrient contents of which are described in Table 1, and had free access to mains water.

Experimental procedure

The experiments were carried out from November 11 to November 14, 2009 from 10.00 to 14.00, using 22 rats, with no time interval between sampling and processing.

Table 1. The measured maximum forces, cross-sectional areas and estimated failure stresses.

Number of specimen	Maximum force (N)	Cross-sectional area (mm^2)	Failure stress (MPa)
1	47.2	2.61	18.1
2	45.2	2.86	15.8
3	30.3	1.90	15.9
4	45.5	3.73	12.2
5	35.2	2.48	14.2
6	46.6	2.55	18.3
Mean value	41.7	2.69	15.7
Standard deviation	7.1	0.60	2.3
Relative error (%)	17.1	22.35	14.8

The animals were euthanized with an overdose of ether and a skin incision at the site of gastrocnemius muscle was elaborated. The hind limb was disarticulated at the knee joint and the skin and fascia were removed, in order to expose the muscle-tendon-bone unit. The tibia was removed at the ankle, and the forefoot was removed from the midfoot, thus leaving the midfoot and the hindfoot attached to the Achilles tendon. Special surgical care was taken in order to avoid injury of the Achilles tendon, while separating the soleus and flexor digitorum superficialis muscle-tendon units.

Experimental set-up

For the purpose of the present study a modified cryo-jaw was used and was based on that proposed by Wieloch et al. (*2004*). The clamping device consists of two separate parts. The first is a pincers-like clamp for bone fixation, while the second is the modified cryo-jaw which comprises of four parts (Fig.1). Parts A and C are identical and, when

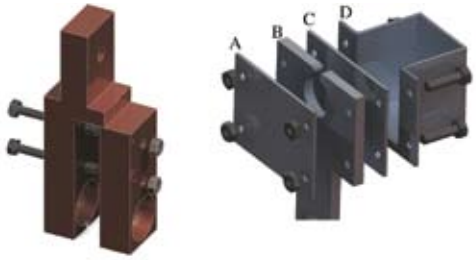

Figure 1. The pincers-like clamp for bone fixation (left) and the modified cryo-jaw (right).

combined with part B, they form a cavity inside which the muscle of the bone-tendon-muscle unit is placed. The specimen is placed in such way that only the muscle is in contact with the surfaces of the jaw. Part D is the cup for the refrigerant (Liquid Nitrogen, L-N). The cryo-jaw is fixed at the inferior site of the load frame and the L-N cup is covered with an aluminium foil sheet to avoid freezing of the tendon from the evaporated L-N.

In order to estimate the volume of L-N needed to freeze entirely the muscle but not the tendon, a number of preliminary tests were performed. The muscle of the specimen was placed inside the cavity of the cryo-jaw which was then fixed on the load frame (MTS MiniBionix 858, MTS Systems Corp., Eden Prairie, MN, USA). The bone was clamped with the pincers-like clamp (Fig. 2). A T-type thermocouple probe was inserted inside the tendon tissue at the transition area between the tendon and the muscle giving real time temperature measurements. Finally a volume of L-N was poured inside the cup, the drop of the temperature of the tendon was recorded and axial displacement was imposed to the pincers-like clamp at a rate of 1 mm/min. The axial force exerted on the specimen was measured using a 50 kgr Instron Tensile Load Cell (Instron, Canton, MA) and the measurements were recorded at a rate of 10Hz. These preliminary tests revealed that pouring 75cm^3 of L-N into the cryo-jaw was enough to freeze most of the muscle to a satisfactory degree in order to withstand loads until failure without any noticeable slippage. At the same time the tendon re-

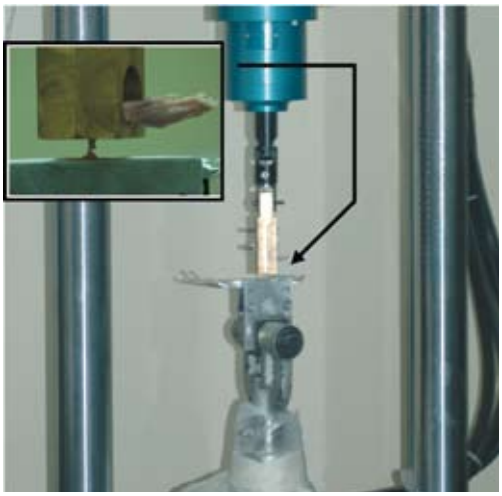

Figure 2. The experimental set-up.

mained unfrozen with a temperature drop of about 10 ºC. During the biomechanical tests, the room temperature was kept constant at 25±2 ºC.

The aforementioned procedure, without the use of thermocouple, was followed for the estimation of the tensile strength of the Achilles tendon of Wistar rats: Six bone-tendon-muscle units were harvested from the right feet of six rats. The units were first submitted to biomechanical testing according to the previously described procedure. After the testing they were submitted to histological analysis and their cross sectional area was determined.

Histological Fixation

Achilles Tendons were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), post-fixed in 1% osmium tetroxide (OsO4), sequentially dehydrated in a series of upgrading concentrations of ethanol followed by propylenoxide and embedded in a Spurr resin mixture. Sections were stained with toluidine blue for light microscopy (Fig.3).

Measurement of the "Effective" Cross-sectional Area

After the fixation, cross-sections of the Achilles tendons, 10μm thick, were obtained at the site of the rupture provoked by the biomechanical testing (Fig.3). Special care was taken to ensure that the whole cross-sectional area of the tendon would appear in each section and that the sections would be absolutely transverse. From each Achilles tendon ten consecutive cross-sections were cut and they were examined using light microscopy. Digital photographs were obtained for each section at a 10-fold magnification. The area of each one of the ten sections was measured using MatLab (MathWorks, Natick, MA, USA) and their mean value was assessed for each specimen.

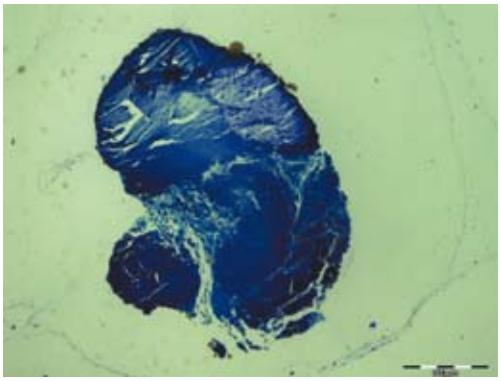

Figure 3. A characteristic cross-sectional section of a rat Achilles tendon after the biomechanical test.

The measured cross-sectional area of each tendon was used for the assessment of the maximal sustained stress of the tendon, based on the fact that, when the tendon is subjected to quasi-static tension, its mechanical behaviour is not affected by its internal fluid pressure (*Herzog*, 2007). In addition the tensile bearing capacity of the liquid phase of the extracellular matrix of the tendon is obviously negligible and therefore it can be excluded from the stress assessment procedure. The cross-sectional area of the dehydrated tendon corresponds to the area of the solid components of the tendon, most of which is collagen. Generally, the collagen content is over 75% of the dry weight of a tendon and up to 99% in cases of extremity tendons (*Nordin et al.*, 2001). Besides collagen the area of solid components consists also of ground substance and a small

amount of elastin. Since these components are the only ones considered capable of bearing loads their cross-sectional area can be considered as an "effective" cross-sectional area of the tendon (A^{eff}). So the tensile fracture strength, f_t of the tendon is assessed by the relation:

$$f_t = \frac{F^{max}}{A^{eff}}$$

where F^{max} is the maximum force recorded during the tensile test.

Results

The temperature measurements in the preliminary tests showed that the tendons were not frozen in any way, as the lowest temperature recorded in the musculotendinous junction was 15±2 ºC. This excludes the possibility that the biomechanical properties of the tendons were affected to such a degree that would render the results unreliable.

The rapid freezing of the muscle alone provided the necessary fixation force capable to sustain significant tension loads up to the fracture of the tendon without slippage and without affecting the mechanical behaviour of the "gage-length" of the tendon. In Fig.4 a characteristic load-displacement curve from the present series of experiments is shown. The three portions, typical for soft tissues under tension, are clearly distinguished: An initial non-linear region ("toe" region) is followed by an almost linear one which eventually leads to a sudden drop of the load due to the failure of the tendon.

The average maximum tensile force (F^{max}) was measured equal to 41.7N with a standard deviation of 7.1N, corresponding to a relative error equal to 17.1%. On the other hand the cross-sectional area of the tendons (A^{eff}) after the biomechanical test were found equal to 2.69±0.60mm^2. Following the procedure previously described, the average failure stress was estimated to 15.7MPa with a standard deviation of 2.3MPa and a relative error equal to 14.8%, almost 13.5% lower than the respective error of the fracture forces. The results for all specimens are presented in detail in Table 1.

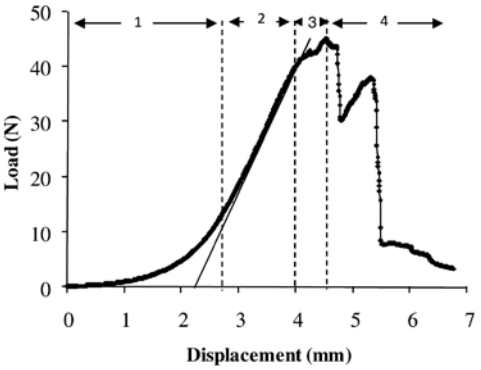

Figure 4. A characteristic Load-Displacement curve from a tensile test of a Wistar rat Achilles tendon. (1) The "toe region", (2) "linear" region, (3) and (4) "failure" regions.

Discussion

Using small laboratory animals for the elaboration of experimental research protocols is of great value for the comprehension of the biomechanical behaviour of soft tissues such as the tendons. The fact that these studies cannot be easily performed *in vivo,* renders the design of a reliable apparatus for *in vitro* testing very important, provided that the biomechanical characteristics of the examined tissue are minimally affected.

It is well known that one of the most important parameters that could significantly affect the biomechanical properties of a soft biological tissue during an in vitro test is the boundary conditions imposed to the specimen. In particular, in the case of the Achilles tendon, gripping is one of the most challenging and important experimental parameters mainly due to its extremely small size.

Usually gripping a specimen in a tensile test is achieved by compressing it between the plates of a clamping devise generating high frictional forces. But in the case of tendons, which are soft, wet, collagenous tissues, strong frictional forces are difficult to be developed because the friction coefficient between the tissue and the material of the clamping devise is very low. One way to increase frictional forces is by increasing the compression force ex-

erted on the specimen inside the clamping device. However in this case higher compression of the tendon leads to dehydration and formation of a water film between the surfaces of the tissue and the grip leading in turn to a significant decrease of the coefficient of friction. Additionally, high compression force could damage the tissue and alter its mechanical properties. Alternatively increased frictional forces can be obtained by modifying the surfaces of the clamping device in order to increase the friction coefficient. Typical example is the study by Viidik, where the metallic surfaces of the grips that were in contact with the tissue were covered with different layers of textiles and paper, but with rather poor results *(Viidik, 1966)*. Relatively good results can be found in the literature for serrated jaw clamps. For example in the study by Cheung et al. (2006) bovine tendons were loaded up to 3000N before slippage occurred. Even with significant high compression of the tissue the jaws weren't able to grip the tendon until failure.

It is clear from the literature that increased compression force on the tendon is inevitable in clamping devices using friction. Unfortunately compression of the specimen can cause large deformation of the clamped part, as well as changes in the initial length of a number of fibres. These alterations can produce an uneven distribution of the load among the fibres. Additionally, since the friction between adjacent fibres is lower than the respective one between superficial fibres and the clamp, a relative slippage of the inner fibres can occur without being noticed. In this case the fibres that lay near the surfaces of the tendon will carry higher loads than the inner ones producing this way a misleading force vs. displacement curve.

An alternative gripping technique is the one based on rapid freezing. This technique was introduced by Riemersa et al. (1982), who froze both the specimen and the clamping device with the use of liquid CO_2. They observed that rapid freezing resulted in a strong gripping of the tendon to the jaws. However this mainly occurs with much bigger tendons, compared to that of rats (e.g. horse tendons). In the case of small laboratory animal tendons this methodology cannot offer sufficient grip of the tendon, as the contact surface is limited.

Wieloch et al. (2004) proposed a modification of the device of Riemersa et al. (1982). Their main idea was to freeze the muscle instead of the tendon, in an appropriately shaped cavity of the cryo-jaw, achieving adequate holding of muscle-tendon units. The methodology described in the present paper is based on the same principle, but also explores some important aspects that had not been studied previously. The use of a thermocouple at the muscle-tendon junction confirmed the fact that the tendon was not frozen; therefore its biomechanical attributes were not substantially changed. In addition the methodology proposed by Wieloch et al. (2004) was elaborated for biomechanical testing at a loading rate equal to 1000 mm/min. However at such a loading rate the tests can not be characterized as quasi-static ones but rather they resemble dynamic testing. The displacement rate used in the present study was much lower (1 mm/min) resulting in a quasi-static biomechanical testing of the soft tissue.

However the main contribution of the present study is the proposal of an innovative technique for the assessment of the fracture stress of tendons based on the histologically-measured cross-sectional area of the tendon. The direct and constant measurement of cross-sectional area during the experimental procedure (e.g. using laser micrometers or miniature LVDTs) is extremely difficult and perhaps unreliable due to the very small size of the specimens and sophisticated mechanisms are required for the parallel transfer of the measuring devices with the continuously elongated tendon. On the other hand the measurement of the initial cross-sectional area of the tendon cannot offer reliable stress data, since it is known that during the biomechanical testing the cross-sectional area of the tendon, which is a hyperelastic material, changes dramatically. Therefore the histological measurement of the "effective" area after the rupture of the tendon appears to be closer to the actual cross-sectional area at the moment of fracture.

Another significant parameter of the experimental procedure is the preconditioning of the specimen. However the nature of the influence of preconditioning on the biomechanical behaviour of a soft tissue is not clear. While it was traditionally considered to be an experimental artifact, recent studies suggest that it is a physical property of the tendon and therefore an experimental parameter itself (*Maganaris*, 2003; *Maganaris et al.*, *2002*). In the present methodology preconditioning was not elaborated due to the risk of de-freezing of the muscle and slippage of the specimen.

The limitations of the methodology proposed here emanate mainly from the nature and the geometrical characteristics of the tested specimens. First of all the strain of the tendon was not determined. The small dimensions of the specimens rendered the utilization of optical strain measurement techniques very difficult and the displacement of the load-frame could not be accepted as elongation without second thoughts. In any case this issue remains available for further study.

Finally, according to the proposed methodology, the measured cross-sectional area corresponds only to the moment of failure. Therefore, it can not be used for the reduction of the entire set of load measurements to the corresponding stresses, but only for the reduction of the maximum force to the fracture stress.

The above observations render the proposed methodology ideal for comparative biomechanical studies of soft tissues in small laboratory animals considering the difficulties encountered using such specimens.

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