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Differential scanning calorimetric examination of the ruptured Achilles tendon in human

N. Wiegand [a], L. Vámhidy [a], L. Kereskai [b], D. Lőrinczy [c,*]

[a] Dept. of Traumatology, University of Pécs, Faculty of Medicine, H-7624 Pécs, Szigeti str. 12, Hungary
[b] Dept. of Pathology, University of Pécs, Faculty of Medicine, H-7624 Pécs, Szigeti str. 12, Hungary
[c] Dept. of Biophysics, University of Pécs, Faculty of Medicine, H-7624 Pécs, Szigeti str. 12, Hungary

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ABSTRACT

The Achilles tendon rupture is a common injury of the foot in middle age and physically active population in Europe. The aetiology of the degenerative changes in the collagen structures of the tendon which could be disposed for the rupture is still not clear. Our hypothesis was that before the injury there is a clear pathological abnormality in the tissue elements building up the Achilles tendon, which is responsible for the disease, and could be monitored besides the classical histological methods by differential scanning calorimetry.

The thermal denaturation of human samples was monitored by a SETARAM Micro DSC-II calorimeter. All the experiments were performed between 0 and 100 °C. The heating rate was 0.3 K/min. DSC scans clearly demonstrated significant differences between the control and ruptured samples (control: T_m = 59.7 °C, $T_{1/2}$ = 1.4 °C and ΔH_{cal} = 8.54 J/g; ruptured: T_m = 62.75 °C, $T_{1/2}$ = 2.6 °C and ΔH_{cal} = 1.54 J/g). These observations could be explained with the structural alterations caused by the biochemical and structural processes.

With our investigations we could demonstrate that DSC is a useful and well applicable method for the investigation of collagen tissue of the human Achilles tendon. We can prove with this method, that the earlier series of microtraumas which result a scar formation in the tendon tissue increases the thermal stability of collagen in ruptured tendon.

1. Introduction

The Achilles tendon rupture is a common injury of the foot in middle age and physically active population in Europe, and there is a continuous increase in the incidence. The aetiology of the degenerative changes in the collagen structures of the tendon which could be disposed for the rupture, is still not clear. Several extrinsic and intrinsic factors have been shown to be associated with this injury. Examples of intrinsic factors are tendon vascularity, gastrocnemius–soleus dysfunction, age, gender, body mass and height, pes cavus, and lateral ankle instability. Extrinsic factors that may predispose to Achilles tendinopathy in athletes are changes in training pattern, poor technique, previous injuries, footwear and training on hard, slippery or slanting surfaces. Excessive loading of tendons during vigorous physical training is regarded as the main pathological stimulus for degeneration. Tendons respond to repetitive overload beyond physiological threshold by inflammation of their sheath, degeneration of their body, or a combination of the two. Whether different stresses induce different responses remains unclear. Tendon damage may even result from stresses within physiological limits, since frequent microtrauma may not allow enough time for repair. Microtrauma can also arise from non-uniform stress within tendons, producing abnormal load concentrations and frictional forces between the fibrils, with localized fibre damage [1].

The degeneration of the collagenous structure of the tendon is a preceding stage of Achilles tendon rupture. The tendon integrity depends on the extracellular matrix metabolism which is regulated by proteolytic enzymes. However, it is unclear which enzymes play a role in tendon rupture. The molecules of extracellular matrix may coordinate morphogenesis, cell differentiation, and most importantly, fibrogenesis in tissue. In the healthy part of the tendon the carbohydrate content is greater than in the ruptured area. In the ruptured area the core protein synthesis is increased, but the glycosaminoglycan production is normal. The tissue in the area of rupture undergoes a marked rearrangement at molecular level [2,3].

The histological examination of the overused Achilles tendon shows a decreased collagen fibres organisation, intensive collagen

* Corresponding author. Tel.: +36 72 536 261; fax: +36 72 536 261.
E-mail address: denes.lorinczy@aok.pte.hu (D. Lőrinczy).

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staining and increased cell nuclei numbers. Immunhistological cell typing suggests that the observed increased cellularity does not include significant inflammatory component, but in secondary an increased numbers of endothelial cells and fibroblasts [4,5]. Various types of degeneration may be seen in tendons, but in the Achilles tendon the usual types are mucoid and lipoid. In mucoid degeneration (i.e. glycosaminoglycan accumulation in the tendon) light microscopy reveals large mucoid patches and vacuoles between fibres. Lipoid degeneration is characterized by abnormal intratendinous accumulation of lipid, with disruption of collagen fibre structure [6].

The interference and polarization microscopic studies described that the ruptured Achilles tendons had significantly smaller collagen fibre diameter than the normal tendons, the fibre diameter being −36% in comparison to their healthy counterparts. Similarly the crimp angle of the collagen fibres was also found to be lower in the ruptured tendon than in healthy, normal tendons. Spontaneously ruptured tendons display focal regions with decreased collagen fibre thickness, decreased crimp angle and disrupted crimp continuity. Microscopic alterations possibly result reduced strength of the tendons and placed them at increased risk of ruptures [7].

More recently, studies have suggested that genetic factors the tenascin C (TNC) gene and the collagen V alpha1 (COL5A1) gene are associated with the manifestation of the Achilles tendinopathy and rupture. Both genes encode for important structural components of tendons and ligaments. The COL5A1 gene encodes for a component of type V collagen, which has an important role in regulating collagen fibres assembly and fibre diameters. The TNC gene encodes for tenascin C, which regulates the tissues response to mechanical load [8].

The therapy of Achilles tendon rupture depends on the clinical manifestation of the injury. Conservative treatment (plaster immobilisation) could choose in the cases of partial ruptures and when the ruptured ends of the tendon can overlap during the plantar flexion of the foot. 80% of the cases need surgical intervention: direct suture of the tendon with open or mini-open (percutaneous) techniques [9,10].

Differential scanning calorimetry (DSC) is a well established method for the demonstration of thermal consequences of local and global conformational changes in biological systems. It has never been applied for the investigation of human Achilles tendon. According to the present study the thermograms may prove and follow the degenerative changes in the collagen structures of the Achilles tendon which could be disposed for the rupture.

Our hypothesis was that in the cases of Achilles tendon rupture the clear pathological abnormalities in the tissue elements building up the tendon can be detected by DSC. Earlier examinations have demonstrated that differential scanning calorimetry (DSC) is a useful and well applicable method for the demonstration of thermal consequences of local and global conformational changes in the organs of the musculoskeletal system. Different authors have demonstrated thermal effects of degenerative processes in various tissue samples [11–15]. Numbers of recent experimental studies (dogs anterior crutiate ligament, horses superficial digital tendon, bovine tail tendon) have demonstrated with differential scanning calorimetry that mechanical overload decreases the thermal stability of collagen in an in vitro model [11,16–21]. A calorimetric examination of the ruptured human Achilles tendon has not yet been carried out on international level.

Our aim was to prove with the examinations that there is a definitive difference in the structure of the healthy (4 samples from healthy cadaver) and pathological (10 samples from ruptured) Achilles tendons, which can be reproduced.

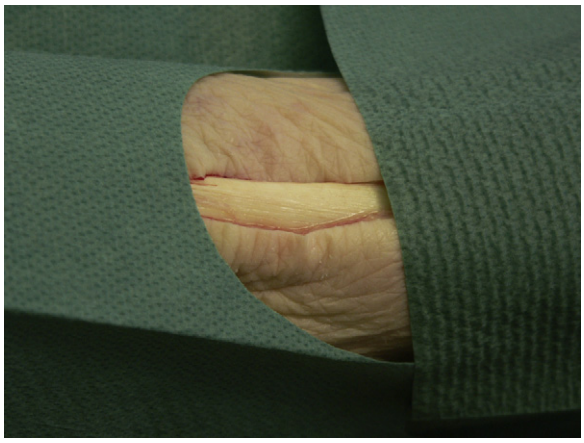

Fig. 1. Intraoperative picture of ruptured human Achilles tendon.

2. Materials and methods

2.1. Sample preparation

The healthy human Achilles tendons were of cadaver origin. We removed Achilles tendon samples (1 cm × 2 cm) from four feet of four individuals (Fig. 1). The donors taken into our study were all under the age of 55 at their death, we considered these persons to be free of any degenerative changes in their joints. We took samples only from feet where any other kind of degeneration or post-traumatic changes of the Achilles tendon could not be verified macroscopically. All the medical interventions were made according to the ethic regulations of the University of Pécs.

The pathologic tendons were derived during surgical treatment of the Achilles tendon ruptures (Fig. 2). During the operations from longitudinal approach over the tendon we prepared the ruptured part of the tendon and cut it out 1 cm × 2 cm long samples from the degenerated part. The ruptured ends of the tendon were connected by a double loop intratendineal 2.0 PDS suture. After the operations we immobilised the extremity in brace for 6 week. We measured 10 ruptured Achilles tendon from two females and eight males being in average 48 years (32–61) of age.

2.2. Histological examination

We removed 1 cm × 2 cm part from the distal and proximal end of ruptured Achilles tendon cut them into two parts. One part has been sent to histological examinations the other underwent DSC investigation. The later samples were put into physiological saline solution and were stored at 4 °C, no longer than 24 h. The samples, subject for histological examination were fixed in 4% formaldehyde, longitudinal and cross-section slides have been made and

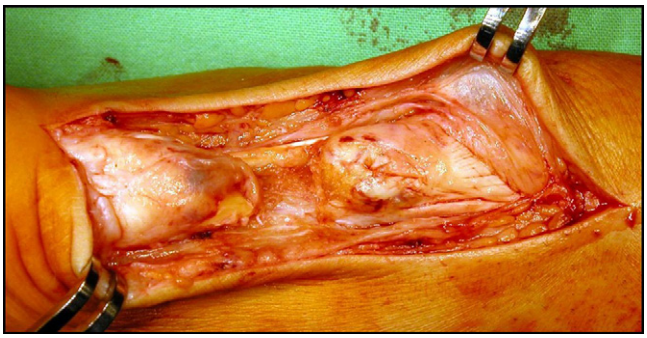

Fig. 2. Picture of an intact human Achilles tendon from cadaver.

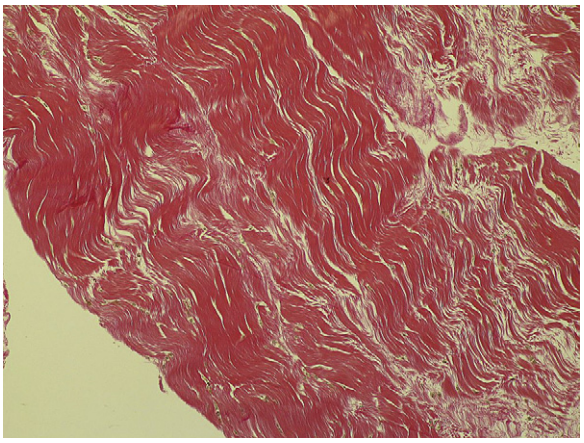

Fig. 3. Histological examination of the healthy human Achilles tendon, normal collagen fibres with picrosyrius stain (magnification 200).

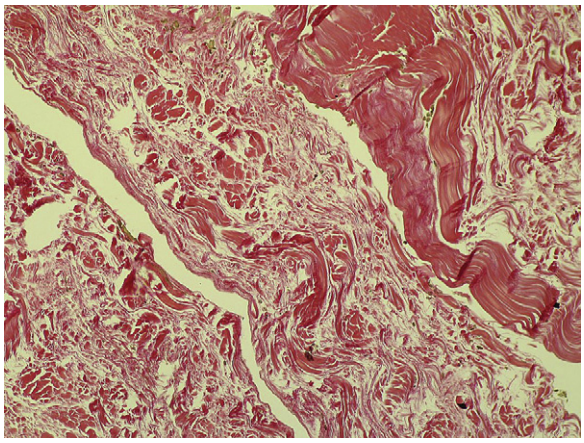

Fig. 4. Histological examination of a ruptured human Achilles tendon: mucoid degeneration, disorganised collagen with disruption of collagen fibre structure with picrosyrius stain (magnification 200).

stained with picrosyrius. Light microscopic control has been performed.

2.3. DSC investigation

The pieces of different samples have been prepared and measured within 6 h of removal. The thermal denaturation of different parts of human samples was monitored by a SETARAM Micro DSC-II calorimeter. All the experiments were performed between 0 and 100 °C. The heating rate was 0.3 K/min. Conventional Hastelloy batch vessels were used during the denaturation experiments with 850 μL sample volume (samples plus buffer) in average. Typical sample wet masses for calorimetric experiments were in-between 100 and 200 mg. RPMI-1640 solution was used as a reference sample. The sample and reference vessels were equilibrated with a precision of ±0.1 mg and there was no need to do any correction from the point of view of heat capacity between the sample and reference vessels. Calorimetric enthalpy was calculated from the area under the heat absorption curve by using two-point setting SETARAM peak integration. The data treatment after ASCII conversion was done by OriginPro 7.5.

3. Results and discussion

Human tendons mostly consist of type I collagen and elastin embedded in a proteoglycan–water matrix with collagen accounting for 65–80% and elastin 1–2% of the dry mass of the tendon according to Ref. [5]. The water content of the tendons is at least 60% [18,22].

Earlier in vitro experimental animal studies (horses digital tendon, bovines tail tendons) demonstrated that the molecular state of collagen is altered by overextension damage, reducing the thermal stability due to intermolecular sliding that liberates specific domains on the molecules, lowering the activation energy for uncoiling. They confirmed that the denaturation temperature is significantly lower in cases of overextended/overused tendons [16–19]. These studies have shown that the calorimetric enthalpy of overextended tendons was not altered by tensile overload, the measured calorimetric enthalpy normalised for dry mass was between 65 and 72 J/g.

With our histological examination we could demonstrate that cadaver Achilles tendon tissues showed no sign of degeneration, regular collagenous structure could be seen (Fig. 3). The pathologic samples showed marked signs of degeneration: decreased collagen fibres organisation, more intense collagen staining, and increased cell nuclei number. The increased number of endothelial cells and fibroblasts represents a biological repair response resulting from repetitive micro-injuries (Fig. 4).

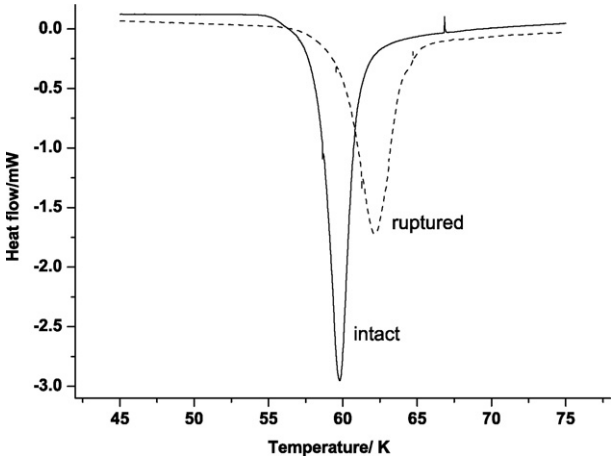

Fig. 5. Thermal denaturation scans of normal and ruptured human Achilles tendon.

According to our knowledge this study is the first in the line of human Achilles tendon rupture research that used thermal analytical method. The results of thermal denaturation can be seen in Fig. 5 and the most important thermal parameters are presented in Table 1. During our investigation we measured the denaturation temperature of the injured and healthy tendons, and calculated the calorimetric enthalpy normalised for the wet mass of the sample. Our data represent an opposite issue than published in the literature, the denaturation temperature of the injured tendons was significantly higher than the healthy samples (Table 1). This could be the sign of smaller amount of bound water in ruptured tissues as a consequence of structural alterations made before the injury. Instead of the structural changes of the overused tendon where the overextension results in intermolecular and intrafibrillar sliding in the cases of Achilles tendon rupture there is a clear pathology

Table 1
The characteristic thermal parameters of the denaturation of Achilles tendons (T_m = melting temperature, $T_{1/2}$ = half width of denaturation temperature, and ΔH is the calorimetric enthalpy normalised for wet sample mass. Data are mean × S.D.).

	Control (n = 4)	Ruptured (n = 10)
T_m (°C)	59.7 ± 0.1	62.75 ± 0.1
$T_{1/2}$ (°C)	1.4 ± 0.05	2.6 ± 0.05
ΔH_{cal} (J/g)	8.54 ± 0.65	1.54 ± 0.11

of earlier series of microtraumas which result a scar formation in the tendon tissue. In the scarified tissue the elements of collagen are disorganised and the level of type III collagen is increased [23], the tissue itself becomes more compact and less cooperative, these could be the reason of the increased half width of denaturing temperature and decrease of calorimetric enthalpy normalised for tissue mass.

Our calorimetric enthalpy at first look seems to be very small in the case of control tendons compared with the recent data of the literature [16]. This could be the consequence of the different heating rates at first (in our experiments it is 0.3 K/min while in [16] it is 5 K/min) and secondly we have normalised on the wet sample mass. If one take into consideration that the intact Achilles tendon contains at least 60% water [22] and the collagen content is about 60% of dry mass according to other observations [24,25], our results (40% is the dry mass, and in it 60% is the collagen then $8.54/(0.4 \times 0.6) \sim 36$ J/g in the case of a cadaver sample) lay in the range of the published data. We have to take into consideration that in the case of in vitro tendon rupture experiments there is no any tissue mechanism to repair the consequences of mechanical overload but in an in vivo Achilles tendon rupture during the "self-reparation" there is a scar formation (it will occupy the place of collagen fibres, therefore will be a decrease in their total tissue mass). This way the results of in vitro and in vivo are not directly comparable.

With our investigations we could demonstrate that DSC is a useful and well applicable method for the investigation of the human Achilles tendon tissue and proved that repetitive microtrauma increases the thermal stability of collagen which could be disposed for the rupture.

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