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Differential scanning calorimetric examination of ruptured lower limb tendons in human

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Abstract The tendon ruptures are serious injuries of the lover limb in middle age and physically active population. While the Achilles tendon rupture is common, the patellar ligament and quadriceps ligament ruptures are an absolutely rare injury. Usually there is no correlation between the velocity of the trauma and the supervening of the rupture. The aetiology of the degenerative changes in the collagen structures of the tendons and ligaments which could be disposed for the rupture are still not clear. Our hypothesis was that before the injury there are clear pathological abnormalities in the tissues of the tendons, which are predisposed for the rupture, 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 $\Delta H_{cal} = 8.54$ J/g; ruptured Achilles tendon: $T_m = 62.75$ °C, $T_{1/2} = 2.6$ °C and $\Delta H_{cal} = 1.54$ J/g, ruptured Quadriceps tendon: $T_m = 64.8$ °C, $T_{1/2} = 1.6$ °C and $\Delta H_{cal} = 1.53$ J/g, ruptured Patellar tendon: $T_m = 63.9$ °C, $T_{1/2} = 1.41$ °C and $\Delta H_{cal} = 0.97$ J/g). These observations could be explained with the structural alterations caused by the biochemical processes. With our investigations we could demonstrate that DSC is a useful and well applicable method for the investigation of collagen tissue of the degenerated human tendons and ligaments. We can prove with this method that the degenerative changes of the tissue elements increase the thermal stability of collagen tissues of the tendons which could be disposed for the rupture.

Keywords Achilles tendon · Quadriceps tendon · Patellar ligament rupture · DSC

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Introduction

Certain similarities can clearly be appreciated between the aetiology and mechanisms of Achilles, patellar and quadriceps tendon ruptures. Both are strong tendons that transmit force bridging at least one joint of the lower limb. When healthy, both require massive forces to be disrupted, and both can be weakened through certain systemic disease processes, steroids and fluoroquinones. The main mechanism of the rupture is indirect trauma. Although clinical diagnosis is easy, ruptures are still frequently missed [1].

The aetiology of the degenerative changes in the collagen structures of the tendon which could be disposed for the rupture is still not absolutely clear. Several extrinsic and intrinsic factors have been shown to be associated with this injury. Examples of intrinsic factors are the age-dependent changes in tendon structure like tendon vascularity, body mass and height, and disorder such gout; diabetes; rheumatic diseases and chronic renal failure are associated causes. Extrinsic factors that may predispose to tendinopathy are changes in training pattern, poor technique and previous injuries.

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 [2].

Spontaneous tendon ruptures are ascribed to recurrent microtrauma resulting from continuous mechanical loading in a critical zone, to muscular imbalance combined with poor coordination as a result of inappropriate training, and also to deteriorating circulation with increasing age [3].

The Achilles tendon rupture is a common injury of the foot in middle age and physically active population (11.3/100,000 per year). The injuries are commoner in males [4]. Quadriceps tendon ruptures are uncommon injuries. Quadriceps tendon ruptures occurred most often in the sixth and seventh decade of life and is probable associated with decreased vasculature relationship between hypovascular zones and patterns of ruptures of the quadriceps tendon [5]. Men were affected six times as often as women. Instead of elder patients the quadriceps tendon rupture in younger patients, can occur in the absence of pathologic tendon degeneration [6].

Patellar ligament rupture usually occurs in patients under age of 40. The injury of this tendon is absolutely rare, only the 3% of all ligament injuries of the knee.

The histological examination of the overused tendons shows a decrease collagen fibres organization, intensive collagen staining and increased cell nuclei numbers. Immunohistological 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 [7–9].

The interference and polarization microscopic studies described that the ruptured tendons had significantly smaller collagen fibre diameter than the normal tendons, the fibre diameter being −36% in comparison to their healthy counterparts. Similar 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 [10].

The therapy of tendon ruptures are usually surgical (80% of the cases): direct suture of the tendon with open or mini-open (percutaneous) techniques [11, 12]. Conservative treatment (plaster immobilisation) could choose in the cases of partial ruptures.

Differential scanning calorimetry (DSC) is a well established method for the demonstration of thermal consequences of local and global conformational changes in biological systems. According to the present study the thermograms may prove and follow the degenerative changes in the structures of the tendons tissues which could be disposed for the rupture.

Our hypothesis was that in the cases tendon rupture the 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 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 [12–16]. Numbers of recent experimental studies (dog's anterior cruciate ligament, horse's superficial digital tendon, bovine tail tendon) has demonstrated with differential scanning calorimetry that mechanical overload decreases the thermal stability of collagen in an in vitro model [12, 17–22]. A calorimetric examination of the ruptured human Achilles, quadriceps and patellar 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 (samples from healthy cadaver) and pathological (samples from ruptured) tendons, which can be reproduced.

Material and method

Sample preparation

The healthy human tendons were of cadaver origin. These samples remain as waste materials when several preparates are dispensed for the bone bank of our orthopaedic clinic. We removed Achilles, quadriceps and patellar tendon samples (1 × 2 cm) from four feet of four individuals (3 males, 1 female). The donors taken into our study were all under age of 55 at their death, we considered these persons to be free any degenerative changes in their joints. We took samples only from feet where any other kind of degeneration or post traumatic changes of the tendons 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, quadriceps and tendon and patellar ligament ruptures (Figs. 1, 2, 3). During the operations from longitudinal approach over the tendon we prepared the ruptured part of the tendon and cut it out 1 × 2 cm long samples from the degenerated part. The

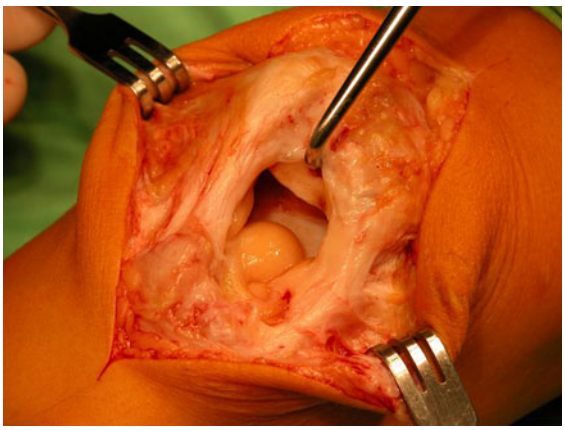

Fig. 1 Intraoperative picture of ruptured human patellar ligament

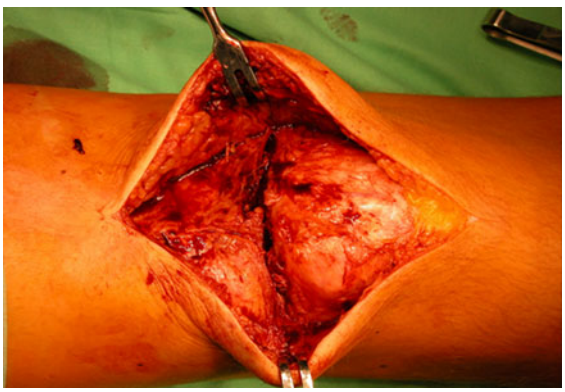

Fig. 2 Intraoperative picture of ruptured human quadriceps tendon

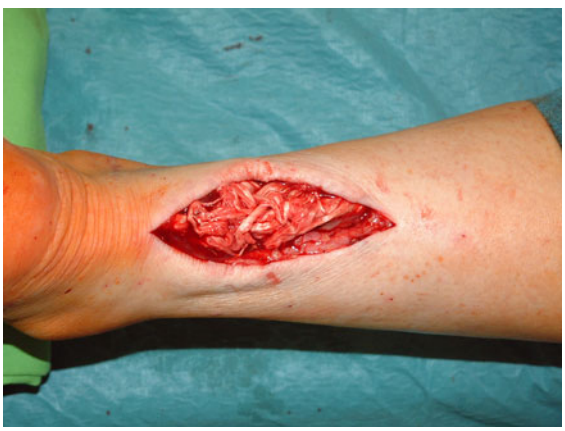

Fig. 3 Intraoperative picture of ruptured human Achilles tendon

ruptured ends of the tendon were than connected surgically. We measured 5 ruptured Achilles (1 female, 4 males), 5 quadriceps (1 female, 4 males) tendon, and 4 patellar ligaments (4 males) the average age were 48 (32–61) years.

Histological examination

We removed the tendons as one piece and longitudinally cut them into two parts. One part has been sent to histological examination the other underwent DSC investigation. The later samples were put into physiological saline solution and were stored separately at 4 °C, no longer than 6 h. The samples subject for histological examination were fixed in 4% formaldehyde, longitudinal slides have been made and stained with haematoxylin and eosin. Light microscopic control has been performed with Nikon Eclipse 400 microscope.

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.

Result and discussion

According to our knowledge this study is the first in the line of human lower limb extensor tendons rupture research that used thermal analytical method. The aim of our study was to compare the histological and thermal parameters of the intact and ruptured tendons of the lower limb, and to identify any discrepancy or conformity of the results from different type of tendons.

Earlier 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 [17–20]. These studies have shown that the calorimetric enthalpy of overextended

tendons was not altered by tensile overload, the measured calorimetric enthalpy of dry mass were between 65 and 72 J/g.

With our histological examination we could demonstrate that cadaver tendon tissues showed no sign of degeneration, regular collagenous structure could be seen (Figs. 4, 5.) The pathologic samples of the Quadriceps and Achilles tendons and patellar ligaments showed a marked similar signs of degeneration: decreased collagen fibres organization, more intense collagen staining and increased cell nuclei number. The increased number of endothelial cells and fibroblasts represent a biological repair response resulting from repetitive micro injuries.

The thermal denaturation results coincide with histological findings (Figs. 6, 7, 8). 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 calculate the calorimetric

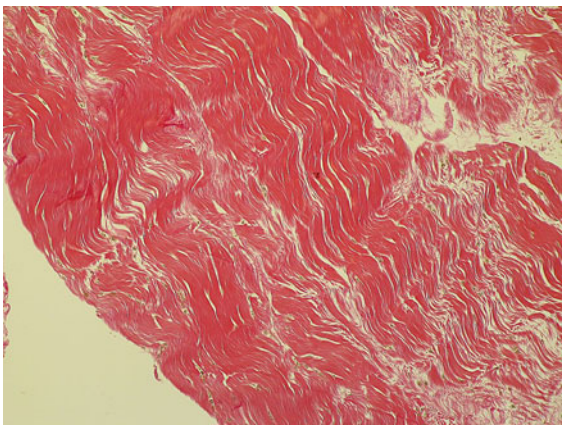

Fig. 4 Histological examination of the healthy human Achilles tendon, normal collagen fibres with picrosyrius stain (×200)

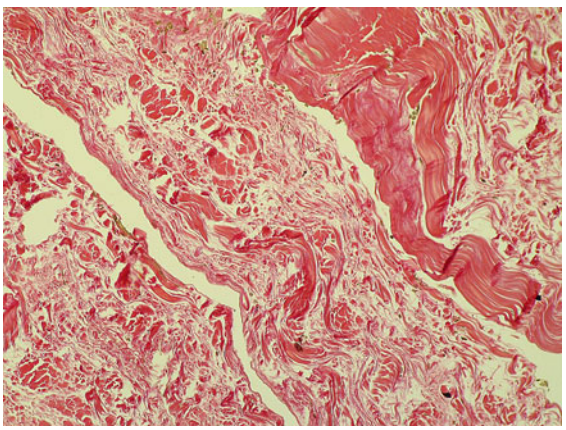

Fig. 5 Histological examination of a ruptured human Achilles tendon: mucoid degeneration, disorganised collagen with disruption of collagen fibre structure with picrosyrius stain (×200)

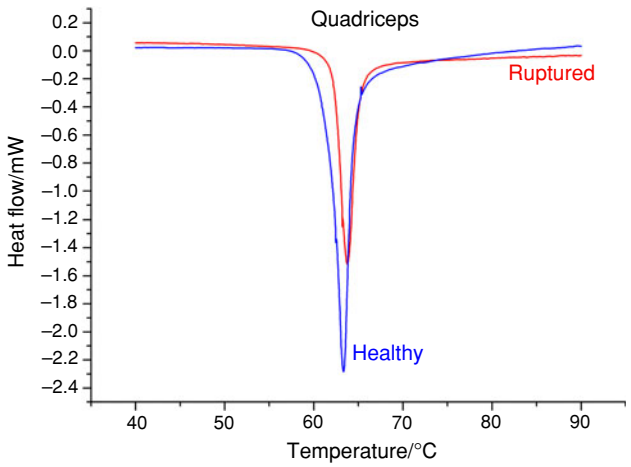

Fig. 6 Thermal denaturation scans of intact and ruptured human quadriceps tendon

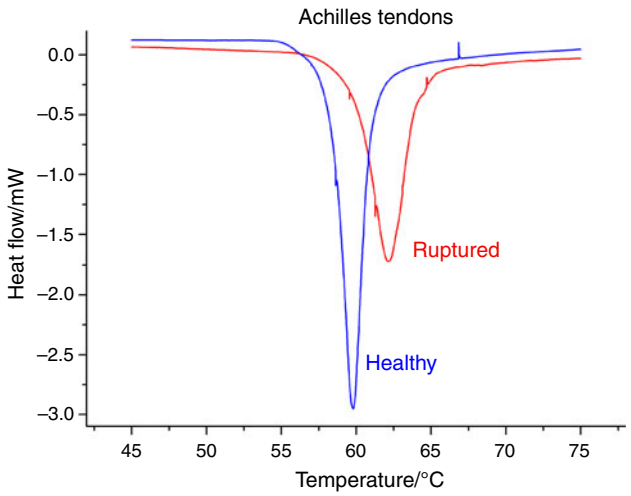

Fig. 7 Thermal denaturation scans of intact and ruptured human Achilles tendon

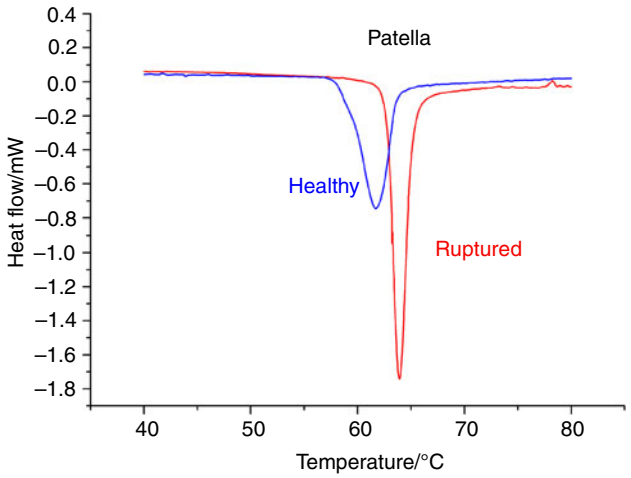

Fig. 8 Thermal denaturation scans of intact and ruptured human patellar ligament

Table 1 The characteristic thermal parameters of the denaturation of intact and ruptured human Achilles, quadriceps and patellar tendons (mean ± SD)

	Achilles		Quadriceps		Patellar	
	Control ($n = 4$)	Ruptured ($n = 5$)	Control ($n = 4$)	Ruptured ($n = 5$)	Control ($n = 4$)	Ruptured ($n = 4$)
T_m/°C	59.7 ± 0.1	62.75 ± 0.1	63.3 ± 0.1	64.8 ± 0.1	61.7 ± 0.1	63.9 ± 0.05
$T_{1/2}$/°C	1.4 ± 0.05	2.6 ± 0.05	1.41 ± 0.10	1.6 ± 0.08	2.63 ± 0.08	1.41 ± 0.08
ΔH_{cal}/J/g	8.54 ± 0.45	1.54 ± 0.08	6.27 ± 0.3	1.53 ± 0.07	4.36 ± 0.2	0.97 ± 0.05

enthalpy of the sample wet mass. Our data represent an opposite issues than published in the literature, the denaturation temperature of the injured tendons were significantly higher than the healthy samples. 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 rupture there is a clear pathology of earlier series of microtrauma which result a scar formation in the tendon tissue. In the killed tissue the elements of collagen are disorganised the level of type III collagen is increased [23], the tissue itself became more compacted and less cooperative, these could be the reason of the increased half width of denaturing temperature and decrease of tissue mass calorimetric enthalpy.

As it can be seen on Table 1, the intact Achilles tendon is the most stable, and it has the higher level of enthalpy ($\Delta H_{cal} = 8.54 \pm 0.45$ J/g) than the patellar ligament and quadriceps tendon, and the smallest half width of its DSC scan (1.4 ± 0.05 °C) verifies a more cooperative and compact structure. The melting temperatures of intact tendons do not differ significantly. In the cases of ruptured tendon the melting temperatures did not show significant differences. The ruptured Achilles tendon has a highest (2.6 ± 0.05 °C) and the patellar ligament has the lowest (1.41 ± 0.08 °C) half width which refers to the loosening of internal structure. Both type of ruptured samples had significant lower enthalpy than the intact ones. The most striking effect in ruptured samples is, that the patellar ligaments have a significantly smallest enthalpy ($\Delta H_{cal} = 0.97 \pm 0.05$ J/g) compared to the other ones, it means that the patellar ligaments structure suffered a biggest repetitive damage before the rupture (Figs. 6, 7, 8).

In order to sum up we can say that we have found significant difference between intact and ruptured tendons including Achilles, quadriceps and patellar tendons too. The thermal parameters of both type of intact tendon were almost the same; there were no significant difference between them. In both cases the ruptured tendons had higher melting temperature and significantly lower calorimetric enthalpy than the intact tendons.

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

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