Introduction

It is well known that ligaments and other dense fibrous connective tissues are prone to creep and relaxation in response to continuous mechanical loading. These tissue responses can all be understood to be expressions of strain induced decrease in tissue stiffness (Fung, 1993). Less well known in the fields of manual medicine and movement therapies is the seemingly opposite tissue response of strain hardening. In this remarkable tissue behaviour, the application of an appropriate strain plus subsequent rest induces a temporary state of increased tensional stiffness in ligaments, tendons and fascia. Although it has been repeatedly documented and discussed in the biomechanical literature, the mechanism of this phenomenon remains unknown (Rigby, 1964; Viidik, 1968; Frisen et al., 1969; Cohen and McCrum, 1976; Betsch and Baer, 1980; Hubbard and Soutas-Little, 1984; Fung, 1993; Yahia et al., 1993; Har-Shai et al., 1996; Har-Shai et al., 1997).

The involved molecular tissue dynamics could involve changes in matrix water binding as was suggested by Viidik (1980) and Har-Shai et al. (1996). Hydration induced changes in tissue stiffness have already been described for dense fibrous connective tissues (Haut and Haut, 1997; Thornton et al., 2001). Such changes have also been implicated in the possibly related phenomena of tissue creep and stress relaxation (Viidik, 1980; Fung, 1993).

Recent biomechanical investigations have provided strong support for the conclusion that tension transmission across the human lumbar fascia contributes to low back stability (Vleeming et al., 1995; Barker et al., 2004; Colloca and Hinrichs, 2005; Barker et al., 2006). This is an important issue in current back pain research (Cook et al., 2006) and has implications for understanding optimal force transmission through the lower back (Norris, 1993; Dolan et al., 1994; Hides et al., 2008). It is therefore of particular interest that strain hardening was reported to occur in human lumbar fascia in vitro (Yahia et al., 1993).

The authors of that study also observed an apparent contraction in fascia samples held under isometric conditions following stretch and suggested that intrafascial contractile cells may be responsible for this behaviour. In fact, studies published subsequently demonstrate that fresh in vitro pieces of rat lumbar fascia can be provoked to perform active tissue contractions in response to stimulation with pharmacological agents that stimulate intrafascial smooth muscle-like cells such as myofibroblasts (Pipelzadeh and Naylor, 1998; Schleip et al., 2007). Such cells are able to induce isometric contraction of their surrounding matrix in response to pharmacological as well as mechanical stimulation (Hinz and Gabbiani, 2003). Based on these newer findings it has been suggested that active fascial contractility facilitated by intrafascial contractile cells may indeed impact musculoskeletal dynamics by altering tissue stiffness in a smooth muscle-like manner (Staubesand et al., 1997; Schleip et al., 2005).

This study therefore examined the new hypothesis that fascial strain hardening is dependent on cellular contraction. Specifically, the assumption under investigation was the position that strain hardening can be induced in viable pieces of fascia yet not in nonviable pieces of the same origin. Changes in matrix hydration were examined in order to explore an alternative explanation.

Materials and methods

Animals and tissue preparation

Nine BALB/cJ mice weighing 25—35 g (age 10—18 wks) were sacrificed by cervical dislocation after narcosis with CO_2 gas for at least 5 min. Animal treatment and experimental procedures were approved by the local committee on ethics of animal experimentation (Ulm University, Germany). Between surgical dissection of the fascia pieces from the animal and final measurements, the samples were kept immersed in room temperature Krebs-Ringer solution (Gibco, Karlsruhe, Germany) or were frequently sprayed with Krebs-Ringer solution (also at room temperature). Air exposure time without spraying was kept to below 2 min. A surgical knife was used to remove all visible muscle fibres from the fascia. This was performed and checked by inspection with a light microscope using 20× magnification. The time between the death of the animal and recording of the last test with a given tissue was kept to below 8 h. The effective sample size had a length of between 12 mm and 18 mm, a diameter of 1—2 mm and weighed between 45 and 96 mg.

From two of these mice one sample each was taken to serve as nonviable control tissue. In these bundles all inherent cells were killed with five cycles of deep freezing in liquid nitrogen followed by rapid thawing. These bundles were used repeatedly (although not more than once per day) and were stored at −70 °C between tests.

Mechanographic investigations

The lumbodorsal fascia (posterior layer) was dissected and a longitudinal strip was excised from the right side of the lumbodorsal spine along with a second sample from the left side; i.e. two test samples were taken from each animal (Fig. 1). Both endings of the sample were secured with a stainless steel ring having a diameter of 3 mm using mercerised cotton thread which had a diameter of 160 μm and a stiffness of 12,500 MPa under dry as well as wet condition. One ring was fastened to the bottom of the organ bath, the other to a stainless steel rod which was connected to the free arm of an isometric force-voltage transducer (Model FT03, Grass Instruments, West Warwick, RI, USA). This transducer was connected to a PC via a bridge amplifier and an analogue-digital board (Digidata 1200B, Axon Instruments, Union City, CA, USA). The sampling frequency was 200 Hz.

Samples were first suspended in the bath in a slack (relaxed) position. By slowly lengthening the tissue, the first point of a reversible force increase (i.e. a clearly detectable increase which could be reversed by a comparable strain decrease and could also be regained by repetition) was defined as zero strain with zero force. Preliminary tests had confirmed that this method of defining 'zero length' corresponded well with the length of the tissues when stretched out horizontally on a flat wet surface, and that the potential effect of buoyancy of the

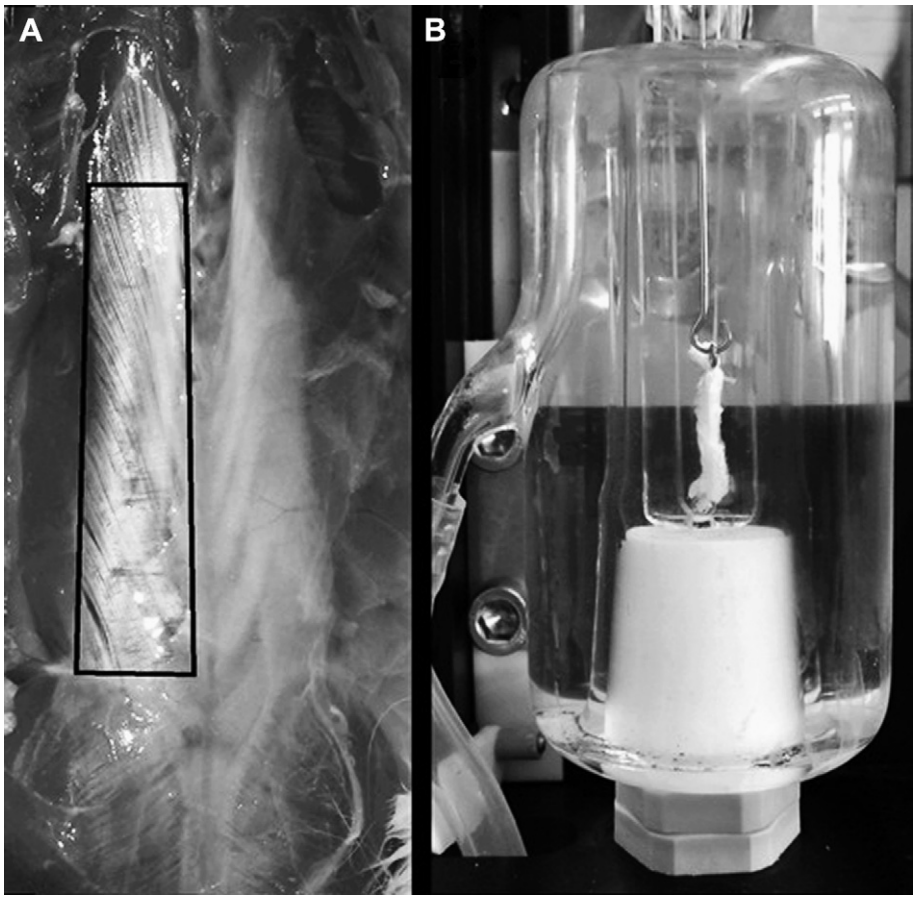

Figure 1 Tissue preparation for in vitro examination. (A) Lumbodorsal mouse fascia during dissection. One long piece of the fascia on the left side of the spine has been marked for removal and further in vitro examination and testing. (B) Tissue bundle during strain application in an organ bath. The upper end of the tissue bundle is connected with a stainless steel rod to an electronic force transducer. The double walled bath container is filled with Krebs-Ringer solution at 35 °C and constantly aerated with 95% $O^2 + 5\%$ CO_2.

fascia bundles in the bath were negligible due to only minimal density differences between the bath solution and the fascia samples. The strips were left in a relaxed state (at zero strain) for at least 15 min before exposing them to mechanical strain as will be described later. All strain changes were conducted at a speed of 0.33% per second as described in other studies (Yahia et al., 1991, 1993).

Strips were exposed to a 4% isometric strain in an organ bath for 15 min followed by 30 min rest at zero strain. The zero setting at rest as well as for a repeated strain application was at the same elongation point as the first strain application. These protocols are similar to the mechanographic tests performed in a previous in vitro study by Yahia et al. (1993) which revealed a strain hardening behaviour in comparable tissue bundles of human lumbar fascia.

Water content changes in porcine fascia

Initial tests had revealed that murine fasciae were less suitable for these additional tests because of their small size (first attempts to measure the water content of mouse fascia revealed incidental changes in the proportion of surface water as even a single extra drop of tissue water could cause notable differences in the wet weight). Therefore larger tissue strips of lumbar fascia from pigs were used for these subsequent examinations. From each animal, one hand-sized piece of lumbar fascia along with the underlying lumbar erector spinae musculature was collected at the local slaughterhouse from four freshly killed female pigs. During transport to the laboratory the tissue was kept in Krebs-Ringer solution at room temperature. Longitudinal samples were dissected, with their axis oriented parallel to the dominant fibre direction. These were further divided and suspended in organ baths in the same manner as the mice tissues described above. In order to lower the variation effects of surface water, larger tissue pieces were taken than for other measurements in this study. A total of 52 tissue bundles were used. Their resulting sizes varied as follows: length between 22 mm and 50 mm, width 7—18 mm, depth 0.5—2 mm, weight 360—2200 mg (mean 1125 mg). The bundles were exposed to isometric strain and subsequent rest as described above. In 24 samples the applied strain was 4% and in another 28 samples 6% was used. The samples were weighed in wet conditions at different stages of this protocol and after drying in an oven for 12 h at 60 °C. The detailed handling procedure was standardized and kept identical throughout

all tests. The relative water content (WC) was calculated from the difference between wet weight (WW) and dry weight (DW) using the following formula:

$$WC = \frac{WW - DW}{WW}$$

Organ bath experiments with hypotonic osmolarity

In order to explore the effect of hydration change on tissue stiffness, six pieces of lumbar fascia from female pigs were used. Stiffness changes in response to a change in the bath solution from isotone Krebs-Ringer solution to distilled water (which is expected to increase tissue hydration) as well as vice versa were examined using the following procedure. Tissues were suspended in the organ bath as was previously described. Following a 15 min period of adaptation to the organ bath environment at zero strain, the strain was increased to 2% and then maintained at this level for 1 min. This was followed by 10 min rest at zero strain. This cycle was performed four times consecutively and the whole procedure was repeatedly applied in different bath solutions. Previous investigations of different strain rates and load/rest periods in Krebs-Ringer solution had shown that this protocol allowed for repeated applications with full recovery towards the initial tension at the end of each application cycle. In the context of these examinations we defined 'stiffness' (k) as the resistance to a deformation of length with $k = dF/dL$; where dF is the change in tension force (i.e. force in axial direction) and dL the resulting length difference with the strain applied in the longitudinal direction of the tissue bundles (Baumgart, 2000). Specifically we measured dF between the beginning and end of the 2% strain elongation process. Stiffness of a tissue in a specific bath solution was determined by taking the mean tension increase of all four tests in that particular bath solution.

Statistics

Data are presented and plotted as means $\pm$ standard deviation (n, number of experiments). Wilcoxon non-parametric tests were used to test for significant differences of mean values. A significance level of $p < 0.05$ was applied.

Results

Isometric strain induces an increase in fascial stiffness

Freshly dissected murine lumbodorsal fasciae were repetitively challenged with 4% isometric strain followed by a period of rest. Responsiveness of fascia was also observed when applying a 6% strain. However, strips were torn in two out of eight samples; so the experiments were continued only with a strain of 4%. In 11 out of the 16 samples the tissues showed a tension increase at the beginning of the second stretch (Fig. 2). Statistical analysis of all bundles shows a significant tension increase of 4.5 ± 5.3 mN or $9.0\% \pm 10.0\%$ ($n = 16$).

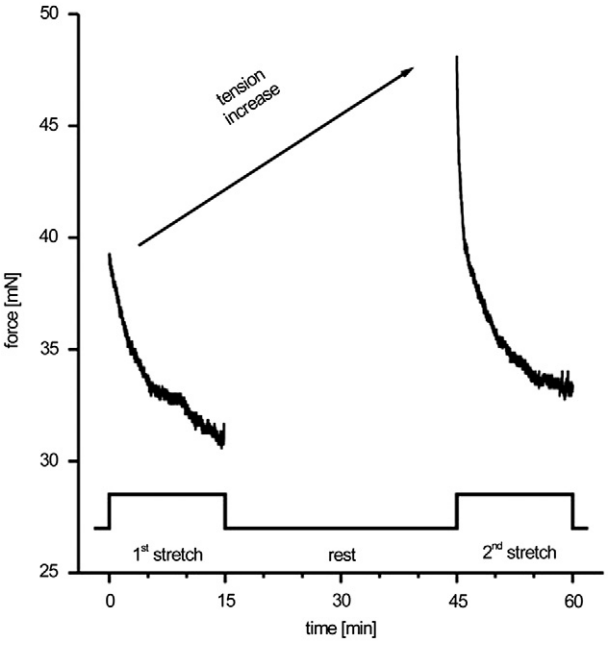

Figure 2 An example of the strain hardening effect of repeated isometric stretches. Reaction of a piece of mouse lumbodorsal fascia in response to repeated strain application. A 4% strain is applied for 15 min, followed by 30 min of rest at zero strain. Lastly the tissue is stretched again. Tissue tension is measured at all times with an electronic force transducer. Note the increase in tension between the first and second stretch indicating an increase in tissue stiffness and resembling the strain hardening phenomenon.

When the same protocol was applied to control samples which had been made nonviable with the described freeze-thaw treatment, the data showed that the strain hardening effect was also present in seven out of the eight nonviable control tests. The tension increase yielded $3.2\ mN \pm 1.8\ mN$ or $7.4\% \pm 5.3\%$ — a statistically significant strain hardening effect. Compared to the fresh samples, there was a tendency toward lower amplitudes of tension increase although not at a level of statistical significance.

Association between strain hardening and loss of tissue water

The first set of experiments revealed that isometric strain lead to a tension increase in lumbodorsal fascia (strain hardening) in the majority of samples. However, contrary to the authors' hypothesis and original expectation, this phenomenon is independent of cellular contraction.

As water makes up the majority of the volume of fascia, this study was extended to include an examination of water content changes of the strained tissues as a possible explanation for fascial strain hardening. Since murine tissue bundles were not suitable for these additional examinations of tissue hydration changes due to their small sizes, larger pieces of porcine lumbar fascia were used for subsequent investigations. The strain protocol was performed analogously to the mechanographic tests described above. Fig. 3 illustrates the response to 4% and 6% strain. The water

content is clearly diminished immediately after the stretch and then gradually returns to baseline levels. Interestingly, an over-recovery or super-compensation was observed in which higher than initial water content levels are achieved if sufficient resting time is allowed. A 6% strain resulted in a faster rehydration process and a more pronounced and significant overshoot after 3 h ($p < 0.05$). Mechanographic investigation were performed post hoc in a few ($n = 3$) smaller pieces of porcine lumbodorsal fascia in which a strain hardening response in porcine fascia was detected as well.

As was shown above, strain hardening accompanies a change of fascial water content. To clarify the potential impact on fascial tension, mechanographic investigations were performed after experimentally increasing the water content. Porcine fascia samples were exposed to changes in osmolarity of the solution. Hypotonicity lead to an increase in fascial water content. Force measurements showed that the samples in the hypotonic organ bath had increased tension compared to isotonic conditions. The difference of $26\% \pm 9.0\%$ was statistically significant ($n = 12$). These results suggest that changes in water content contribute to the fascial strain hardening phenomenon.

Discussion

Interpretation of results

The results of this study confirm the existence of the strain hardening response as was previously described by Yahia et al. (1993) and other authors. It was demonstrated that this strain induced enhancement of tissue stiffness occurred in viable samples of murine lumbodorsal fascia in the applied protocol. Additionally it was shown that this response also occurred with tissues which had been pretreated by deep freezing and rapid thawing. The pretreatment protocol which was used with the control tissues had been shown by other authors to reliably kill all cells in comparable connective tissue samples (Frank et al., 1988; Arnoczky et al., 1992) while leaving the passive viscoelastic tissue properties virtually unchanged (Bechtold et al., 1994; Smith et al., 1996; Moon et al., 2006).

The ability of our investigations to reproduce the same strain hardening feature with nonviable tissues therefore strongly suggests that the strain hardening effect is not caused — at least not exclusively — by active cellular contraction of fibroblasts/myofibroblasts (Schleip et al., 2005).

The results of the reported hydration tests with porcine fasciae revealed that application of strain resulted in a temporary increase in tissue hydration accompanied by an increase in tissue stiffness. While it is possible that the hydration changes are merely a side effect of strain hardening, the results from the additional tests on the influence of a hypotonic solution on tissue stiffness suggests that the increase in tissue stiffness may be at least partially caused by a temporarily altered matrix hydration.

Our choice of fresh mice lumbar fascia as a tissue source (rather than human or porcine tissues) for the original strain stimulation tests was based on the availability of viable tissues and on the reported active contractility of rodent lumbar fascia in vitro (Pipelzadeh and Naylor, 1998). Due to the unexpected results in these original examinations, additional investigations of strain induced hydration changes were added later to this study of fascial strain hardening. For reasons explained above, these additional examinations were performed with fresh porcine lumbar fascia rather than mice lumbar fascia as the small size proved to be unsuitable. No fundamental differences have been shown in the basic viscoelastic response of pig and mice tissue (Viidik, 1980; Komatsu et al., 1998). Although unlikely, it is nevertheless possible that the observed stiffness and hydration changes found in pigs may not exist in a comparable fashion in mice or humans. It is therefore important to stress that care should be taken in interpreting the results from these additional examinations.

As mentioned, the strain level of the tissues needed to be adjusted to the particular animal species in order to avoid sporadic tissue ruptures and to achieve comparable strain effects with the lumbar fascia from these different species. These adjustments probably reflect the different tissue strength of this tissue in humans, mice and pigs. It is also possible that different rest periods would be needed to achieve identical effects (in terms of changes in tissue hydration and/or stiffness) in human tissue as used by Yahia et al. (1993) compared with the tissue used in this study due to differences in viscoelastic properties. Different strain amounts apparently also influence the speed of rehydration. This is exemplified in Fig. 3 (1 h versus 2 h for complete rehydration of the tissues with 4% versus 6% strain) and such differences in viscoelastic properties may also be the basis for the temporal differences between the occurrence of strain hardening in the mice tissues of this study and the hydration measurements in pigs.

Nevertheless, the clear results which were achieved in these tests — both in terms of enhanced water content in the stretched tissues after a sufficiently long subsequent rest period as well as in terms of the stiffness enhancing effect of increased water content — suggest that a similar tissue behaviour may occur with human fascia in vivo. Indeed, nuclear magnetic resonance imaging shows that water is extruded from the Achilles tendon after loading and the subsequent rehydration depends on several factors, among them the amplitude of stretch (Helmer et al., 2006).

General clinical implications

Thus far only Viidik (1980, p. 243) addressed the possible in vivo implications of strain hardening: '*These changes…, especially strain hardening and decrease of viscosity, are from a functional point of view beneficial. They contribute to a more precise transfer of muscular force through the tendon and could well also take place during limbering up routinely undertaken before athletic exercises.*' While this comment was made in reference to a reported strain hardening response in rabbit anterior cruciate ligament and rat tail tendon (involving different strain/rest settings than were used in this examination), it is nonetheless justified to explore its possible applicability to the tissue responses observed in this study.

Since tissue hydration dynamics in vivo could be significantly different than in vitro, verification of these obser-

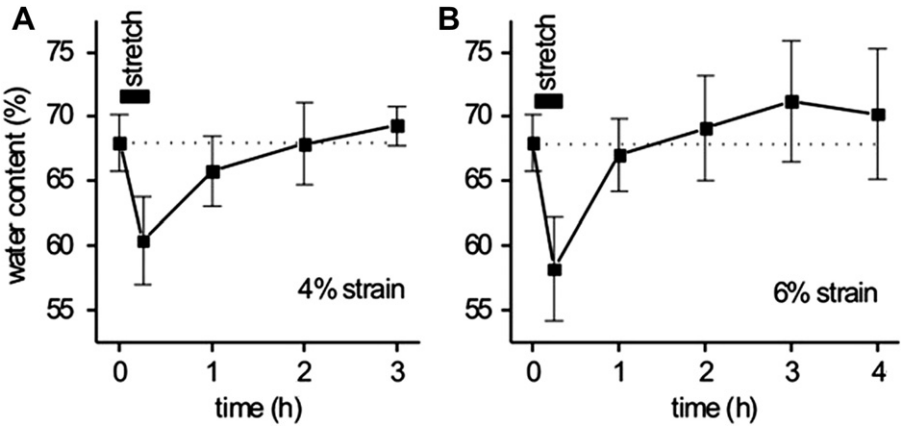

Figure 3 Changes of water content following 15 min stretch. (A) shows mean water content and standard deviation of porcine lumbar fascia samples ($n = 25$) after a 15 min challenge of 4% strain. In (B) the same procedure is applied with a higher strain (6%) and the resting period has been extended to a total of 4 h.

vations under in vivo conditions are needed. If the observed strain hardening phenomenon can be confirmed to also occur in human fascia in vivo, then it is reasonable to expect that similar tissue responses may occur not only in lumbar fascia but also in other dense collagenous connective tissue involved in joint stabilization such as joint capsules, ligaments, retinaculae, aponeuroses and tendons.

Perspectives for bodywork, sports and movement therapy

Myofascial stretching — as is often practiced in yoga therapy and in sports medicine — involves an elongation of tendons, aponeuroses and other dense fibrous connective tissues (Chaitow, 2003; Park et al., 2011). A similar tissue stretch could also be induced by some manual myofascial therapies (Barnes, 1997; Chaudhry et al., 2008, Day et al., 2009). Such tissue loading is capable of inducing a temporary decrease in tissue water content, which has been shown to contribute to alterations in tissue stiffness (Helmer et al., 2006). The results of the present study indicate that during the tissue loading an extrusion of tissue water can be expected and that this tends to contribute to a temporary decrease in tissue stiffness (i.e. tissue softening) immediately after the stretch. While there is little known about the continued development of tissue hydration during subsequent minutes and hours after the tissue elongation, the findings of this study suggest, that during this recovery period a gradual rehydration of the tissue can be expected, which tends to be associated with a gradual regaining of the initial tissue stiffness.

The reported results also suggest that such tissue loading procedures could eventually induce a period in which the tissue stiffness increases beyond the original state — provided that the amount of tissue strain is high enough and that the duration of the subsequent resting period is sufficiently long. Interestingly, a common sport injury prevention regime involves repetitive active stretching followed by periods of seated resting (Green et al., 2002). A study by Green et al. (2002) demonstrated that this sequence of preparation tends to augment lumbar spine stiffness. While the findings of this study could be also related to muscular activation (Solomonow et al., 1998), other studies report that prolonged sitting alone (without prior active loading) tends to induce a stiffness decrease in paraspinal passive tissues (Rogers and Granata, 2006). However the findings reported here suggest that it may not be the sitting posture but rather the sequence of active loading plus subsequent rest which induces a temporary strain hardening in paraspinal tissues.

Future in vivo studies are needed to determine if and under what circumstances the in vitro changes in tissue hydration reported in this study also occur in living bodies. These studies could possibly benefit from recent advances in ultrasound elastography (Huang et al., 2005) and (nuclear MR imaging (Helmer et al., 2006)). Such studies could have implications for a better understanding of the effects of various stretching routines as well as of myofascial manipulation on viscoelastic tissue properties. Further understanding of the strain hardening phenomenon which was observed in this study could also be relevant to the prevention of low back instability in occupational medicine (Cook et al., 2006). Moreover, it offers potential implications for sports-related injury prevention as well as for the enhancement of sports performance (Dolan et al., 1994; Hides et al., 2008).

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