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Doctoral dissertation

Collagen structures from cell culture to intact tendon

SUMMARY

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Co-promoters: Dr. Frantisek Lopot | Charles University
Prof. Virginie Bito | Hasselt University

Annotation

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Promoters:	Assoc. Prof. Karel Jelen | Dept. Anatomy and Biomechanics | Faculty of Physical Education and Sport | Charles University Prof. Marcel Ameloot | Hasselt University / transnational University Limburg
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This summary gives a brief overview of what this PhD thesis (doctoral dissertation) involves and does not explain the topic in detail. It mainly lists the main stages and result using bullet points. For full information, search the chapters in the thesis. The thesis contains seven chapters. Chapter 1 summarizes the knowledge on collagen synthesis, the role of collagen in tendon, the most common methods for collagen visualisation, and collagen mechanical properties. Chapter 2 introduces some methods for quantitative and qualitative collagen identification *in vitro* and highlights the importance of hydroxyproline for second harmonic generation microscopy. Chapter 3 clarifies the impact of sample fluorescent immunostaining to collagenous structures in microtome sections and compares the results with the label-free microscopy methods. Chapter 4 presents an objective automatic analysis for returning the collagen fibre orientation regularity from a single microtome section image. Chapter 5 provides some information on the intact tendon samples and development of the crimp pattern with age. Chapter 6 covers the area of the crimp pattern response to mechanical loading in the intact tendon samples. In addition to that, the change in the helical pitch angle for load-free and stretched intact tendon samples was measured as well as the amount of cross-linkers in tendon. For this purpose, it was designed a miniaturized uniaxial tensile testing device that enables to conduct mechanical tests and microscopy simultaneously. Finally, in Chapter 7, the most important results are summarized and further perspective on the connective tissue topics is offered to the reader.

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Abstract

Type I collagen acts as an extracellular and intracellular marker that reflects tissue properties. To validate these properties accurately, an *in vitro* model with cardiac valve interstitial cells was cultured under two different conditions; with and without ascorbic acid. The cultures were then evaluated by the rate of newly synthesised collagen; DNA content for cell growth; expression levels of collagen gene; fluorescence, confocal, and label-free second harmonic generation (SHG) microscopy. The results indicated that the culture with ascorbic acid reached higher cell growth and collagen deposition, although the expression levels of collagen gene stayed similar to the culture without ascorbic acid. The fluorescent microscopy was positive for collagen fibres in both the cultures. Visualization of only extracellular collagen returned a higher correlation coefficient when comparing the immunolabeling and second harmonic generation microscopy images in the culture with ascorbic acid. Lastly, it was proved that the hydroxyproline strongly contributes to the second-order susceptibility tensor of collagen molecules, and therefore the second harmonic generation signal is impaired in the culture without ascorbic acid.

After evaluation of the methods on the VIC model, the microtome sections from a 31-month-old rabbit Achilles tendon were compared using fluorescent and polarized SHG microscopy. The fluorescently stained microtome sections appeared fuzzy with no details of fibres even under polarized light microscopy. On the other hand, polarized SHG microscopy revealed individual fibres with information on the local orientation. Thus it is possible to limit the sample processing only to microtome sectioning when using SHG microscopy. To examine the influence of microtome sectioning, the tendons from newborn (~7 days) to elderly (~38 months) rabbits were imaged with SHG microscopy and compared by an automatic algorithm. The results plotted in correlogram indicated that after sectioning, the collagen fibre pattern is disrupted for the younger animals. For the older animals, the pattern remained straight, consistent with the longitudinal axis of tendon. This finding was confronted with the dissected intact rabbit tendons.

The intact rabbit tendons from all the animals possessed a strong global directionality, which confirms that the changes in the fibre orientation are triggered by sectioning. However, there was a significant difference in the tendon crimp pattern appearance. The phenomenon can be described by a sinusoidal wave. The crimp amplitude (A) and wavelength (Λ) start at very low values ($A = 2.0 \pm 0.6$ μm, $\Lambda = 19 \pm 4$ μm) for newborn animals. Both quantities are

increased in the sexually mature animals. When the animals are fully mature the amplitude decreases, and therefore it is highly difficult to establish the increasing wavelength.

The age effect was also chosen as an independent variable when investigating the micro-environment, helical pitch angle and mechanical properties of the tendons. It was assumed and proved that the change of micro-environment with age is reflected by the long-lifetime component τ_2 when measuring autofluorescence lifetime of tendon. It was revealed that τ_2 significantly increases with age. On the other hand, the results on the helical pitch angle of the collagen molecule provided no evidence to reject the null hypothesis when measured by polarized SHG microscopy. Similar results were obtained when the helical pitch angle of the collagen molecule was measured under mechanical load, however, the crimp pattern disappeared at minimum resolution of the force sensors. Finally, the tendons were also stressed under uniaxial load being observed by a standard CCD camera. This tensile test returned a high variability data and no statistical difference between the 4-month-old and 38-mont-old animals.

Introduction and objectives

1.1 Collagen, tendon

Collagen is present in organs in order to provide a biomechanical stability, and it also influences the biochemical activity as the extracellular matrix (ECM) regulates the cellular behaviour and cellular differentiation[1]. The role differs among connective tissues from being a supporting structure (type I) to a regulator (type III, type V)[2]. In general, collagen actually forms 1/3 of the total extracellular protein number, and its fibril-forming types I, II, III, V and XI (XXIV, XXVII[3]) are the most abundant types[4]. Type I collagen mostly occurs in bone, ligaments, tendon, skin, cornea, dentin, lung, and vasculature[5-7]. The associated fibrillar types, for example, type III and V usually coexist[5] with type I, and the type ratios are often analysed as markers of optimum composition[8,9]. The final fibril formation is strongly influenced by the types of tissue. For instance, in tendon, type I collagen fibrils are aligned parallel to each other but in skin the pattern follows more isotropic orientation. The fibrillar orientation, diameter, and mass in tissue is not stationary and remodel during ontogeny[10]. The remodelling is strongly related to the matrix metalloproteinase (MMP) family that can unwind and cleave the triple-helical structure in normal and abnormal tissue turnover[11]. Therefore, the properties of tissue change with disease, physical activity, or age.

The physiological changes in collagen tissue with age differ among hierarchical levels. The changes at the molecular level are frequently associated with the collagen types ratio, cross-link formation, and cross-linkers ratio (enzymatic vs. non-enzymatic cross-linking)[12,13]. On the other hand the changes at the upper hierarchical levels, for example, fibres in tendon, are projected in the typical structural crimp pattern[14-16], the origin, development, and function of which is still a subject of discussion[15,17]. To a certain extent, these changes stabilize the network and improve many of mechanical properties at certain hierarchical levels, but, for instance, extensive cross-linking with increasing age (particularly non-enzymatic cross-linking) often causes several drawbacks to the tissue. The fibre-tissue becomes stiffer[18] and more brittle[19], the structural ordering is modified[20], the speed of extracellular matrix remodeling and collagen turnover is reduced[21,22], and the healing ability and injury resistance is also decreased[12]. However, for all that, there is no general agreement[23].

It is obvious that the aging mechanism is very complex and its impact on collagen tissue is still not fully understood. To quantitatively and qualitatively examine collagen structures in

aging tendon, first, a wide pallet of methods was applied on *in vitro* model with ascorbic acid and without ascorbic acid. Second, a common sample preparation method of immunolabeling was confronted with the results acquired by a label-free non-linear visualization method (second harmonic generation microscopy). Third, the influence of microtome sectioning was stated together with the crimp pattern development in intact tendon. Finally, the tendon's micro-environment was analysed by fluorescence lifetime imaging microscopy, the tendon's helical pitch angel was studied thanks to polarized second harmonic generation microscopy at load-free and stretched conditions, and the mechanical tests were conducted in both macro (CCD camera) and micro (SHG microscopy) set-up.

1.2 Objectives of the thesis

The main objective of this thesis was to identify the changes in connective tissue (tendon) due to sample processing and aging and preferably to use label-free methods. The individual goals are listed below:

- Confront the sensitivity of immunolabeling and label-free microscopy to identify collagen structures in *in vitro* model cultured with and without ascorbic acid.
- Prove that hydroxyproline is an indispensable structure for the second-order susceptibility tensor of collagen molecule.
- Compare imaged structures in dense connective tissue using immunolabeling and label-free microscopy.
- Automatically detect and evaluate the orientation regularity of collagen fibres in microtome sections from tendons dissected from animals of different age.
- Map the development of the crimp pattern in tendon and compare the data with the results of microtone sections.
- Identify the mechanical differences between tendons from animals of different age and analyse the response of the crimp pattern to mechanical loading.
- Detect the changes of the collagen helical pitch angle in respect of age and mechanical loading
- Investigate the collagen micro-environment for cross-linking dependency on age.

Material and methods

Materials and methods are thoroughly reported in each Chapter.

2.1 Sample source

- Porcine cardiac valve interstitial cells (VICs) were isolated from pigs, seeded in passages, incubated, and cultured with and without ascorbic acid.
- The rabbit Achilles tendons (*tendo calcaneus communis*) from New Zealand white, Czech Spot, and Belgian Hare crossbred animals of different age.

2.2 Sample processing

- Multiple cell culture harvesting, washing, fixing, staining with primary and secondary antibody.
- Tendon microtome sectioning, intact tendons.

2.3 Data acquisition

- DNA isolation, reverse transcription, real-time PCR.
- Wide-field fluorescence microscopy, single photon confocal microscopy, two-photon excitation microscopy, second harmonic generation microscopy, polarized second harmonic generation microscopy, fluorescence lifetime microscopy.
- Macro uniaxial tensile test with CCD camera as extensometer, micro uniaxial mechanical test with second harmonic generation microscopy as extensometer.

2.4 Data analysis

- Newly synthesised collagen, DNA content for cell growth, expression levels of collagen gene.
- Image correlation, collagen fibre orientation regularity, correlogram, crimp pattern parameters, helical pitch angle at load-free state, and fluorescence lifetime of tendon.
- Stress-strain curve, crimp pattern behaviour under mechanical load, helical pitch angle under mechanical load.

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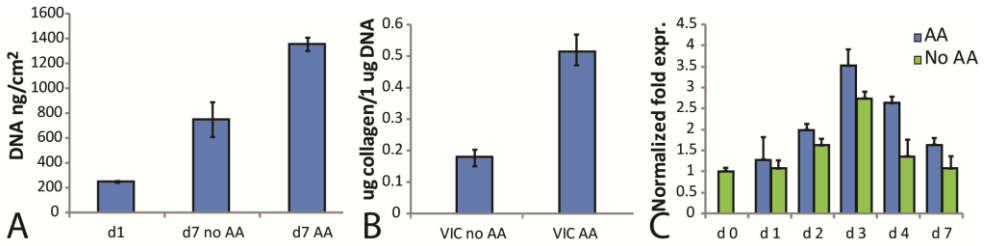
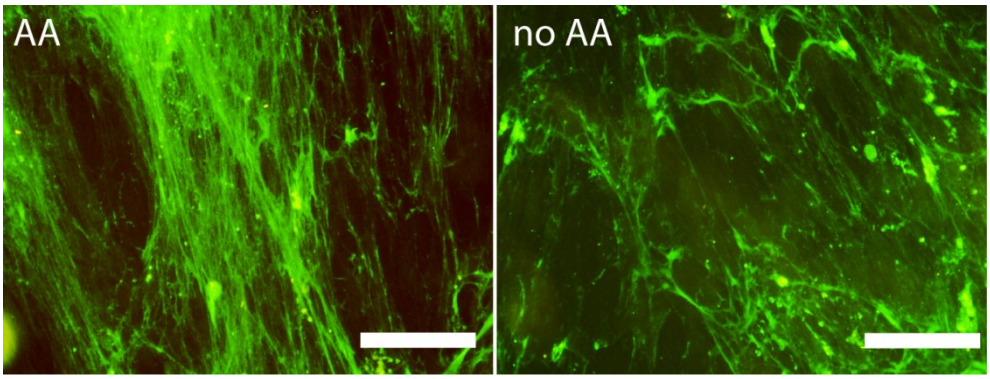
Results

3.1 In vitro – valve interstitial cells culture

- The DNA amount showed that the cell growth was significantly higher in the cell culture with AA compared to the one without AA (Figure A). The amount of collagen deposited in the cell layer was also significantly higher compared to the cell culture without AA after seven days (Figure B). On the contrary, the expression levels of type I collagen gene reached similar values for both the cell cultures during seven days (Figure C).

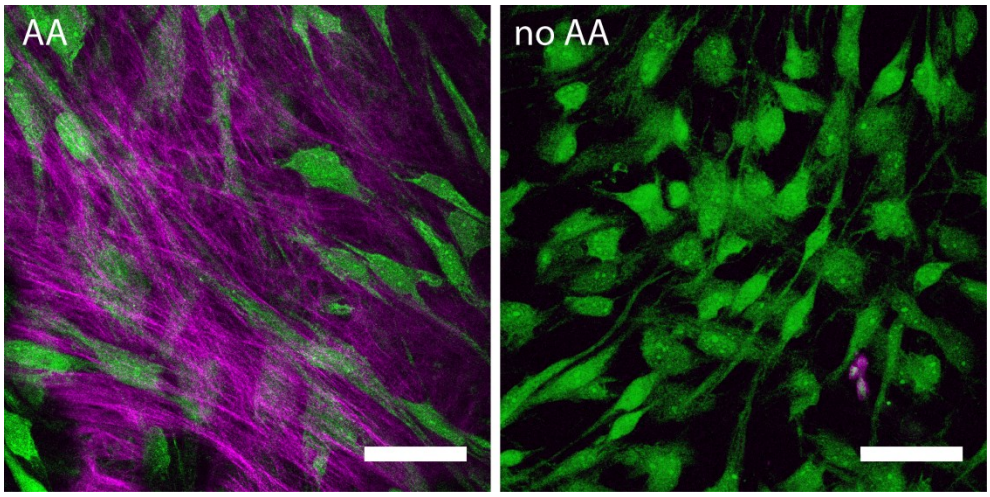


- The fluorescent images revealed a coherent layer of type I collagen in both the cell cultures with and without AA.



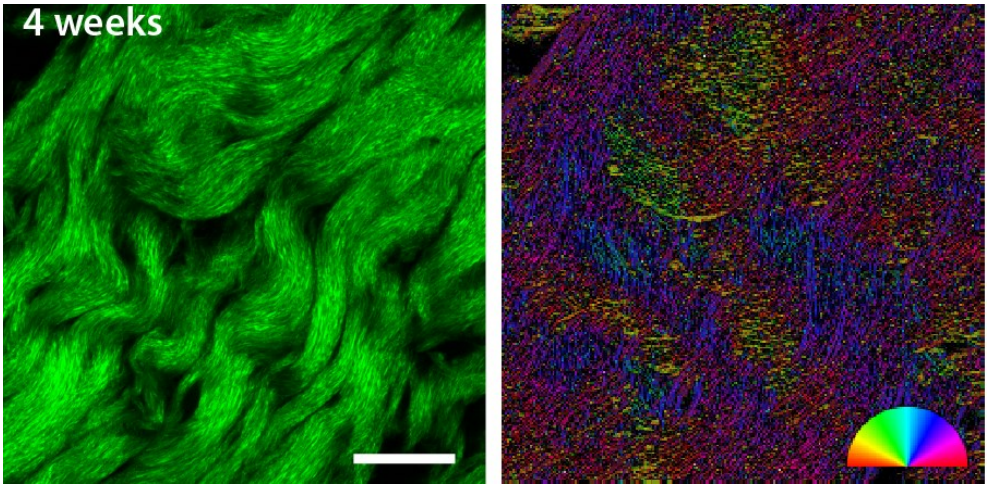
- Only extracellular type I collagen fibres were revealed in the non-permeabilized cell culture, however, the staining appeared weaker. In both staining approaches, i.e. permeabilized and non-permeabilized, the SHG signal was detected, but for the non-permeabilized cell culture group the SHG and TPEM images returned a significantly higher correlation coefficient that indicated a moderate/large positive linear relationship (0.43 ± 0.13 vs. 0.19 ± 0.15; mean ± standard deviation).

- The images with counterstained cell's cytoplasm revealed the presence of the cells in both the cell cultures; however, the SHG signal was clearly detected only in the cell culture with AA.

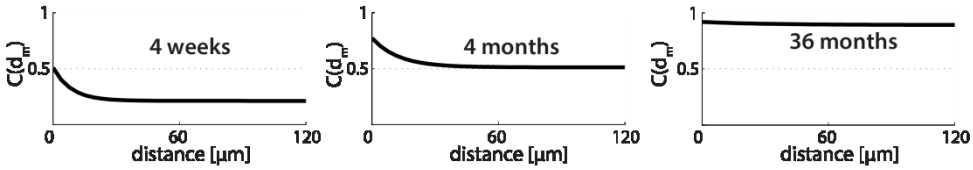


3.2 Ex vivo – rabbit microtome sections

- The images of unstained microtome sections indicated that the collagen structural organization is age dependent. The images of microtome sections from the young animals seemed to have less regular pattern (Figure left) of collagen fibres then the sections from the older animals. The oldest animals possessed the collagen fibres of a straight flat pattern. To express the organization in the microtome sections quantitatively, the orientation of fibres was calculated by the algorithm in Section 4.2.3 and then visualized as the pseudo HSV colour coded images (Figure right).

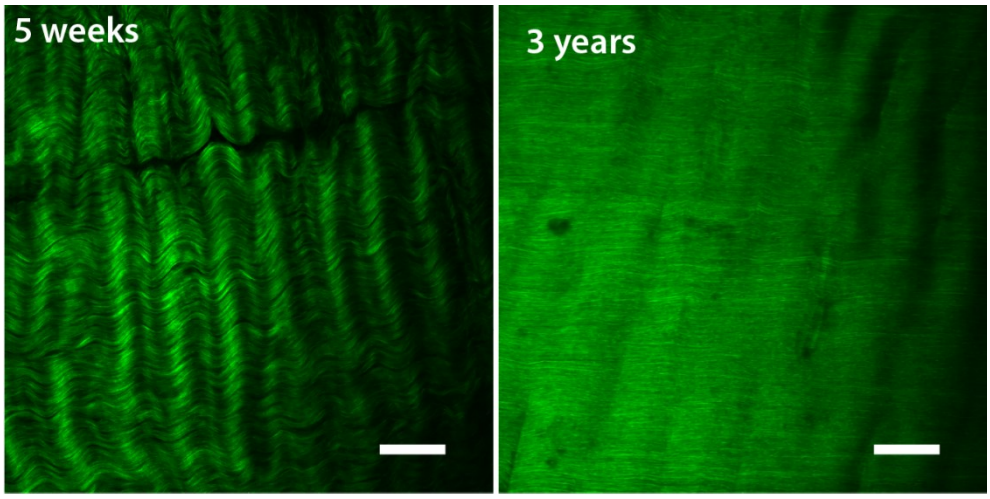


- The orientation regularity is represented by a converging graph in correlogram. The correlograms indicated that the fibres in the microtome sections from the very young animals change directions rapidly with distance and these changes do not follow a periodic, regular, crimp pattern. On the other hand the fibres in microtome sections from the older animals are perfectly aligned within the longitudinal axis of the tendon, i.e. the value in correlogram is close to one and does not change with distance.

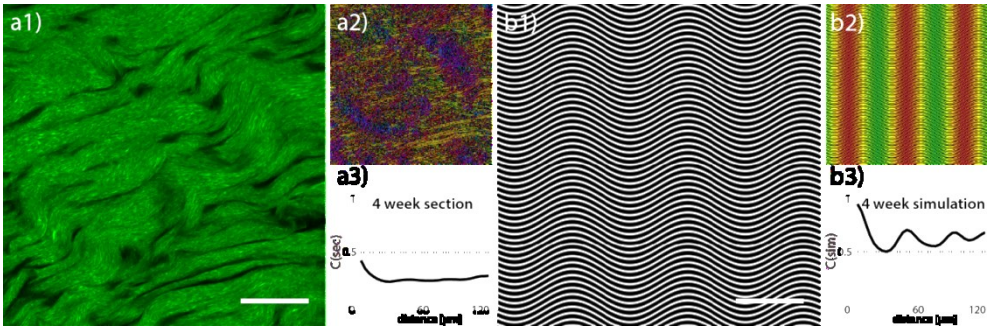


3.3 Ex vivo – intact tendon measurement

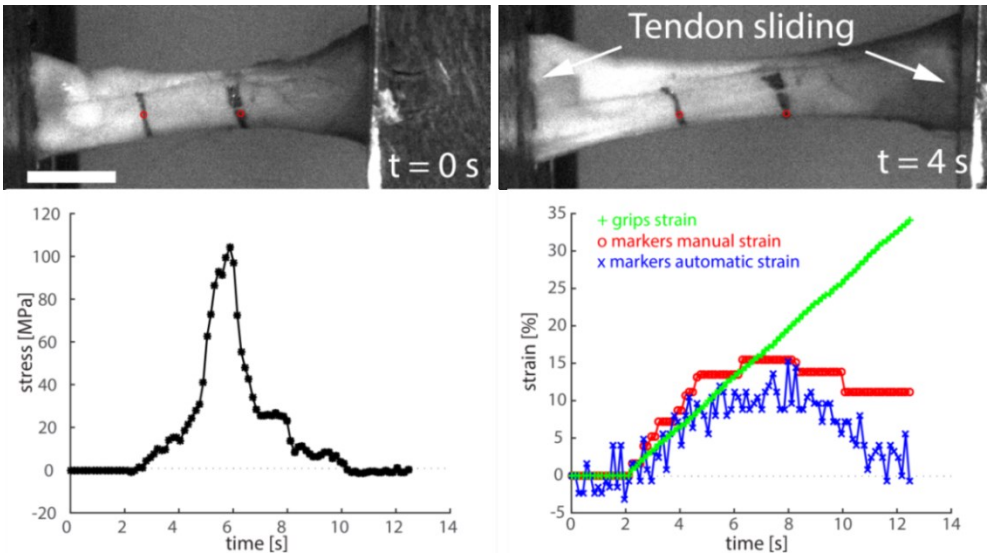
- The global collagen orientation of collagen fibres is predominantly arranged parallel to the longitudinal axis of the tendon in the intact samples. This axis is thought to be the main load bearing axis, and the global orientation is naturally age-independent. In contrast, the local collagen orientation is highly age-dependent. The regular collagen crimp pattern propagates at a certain height/amplitude and periodicity/wavelength through the tendon.



- For 4-week-old animals, the fibres in the microtome section (a1) change the orientation randomly and possess a little regularity (a2). On the contrary, the simulation preserves the crimp pattern (b1). The difference is also detectable by comparing the correlograms (a3, b3). The mean percentage difference (MPD) returns the value of 46 % for the 4-week-old group. For the 36-week-old animals the MPD reaches only 4.3 %.

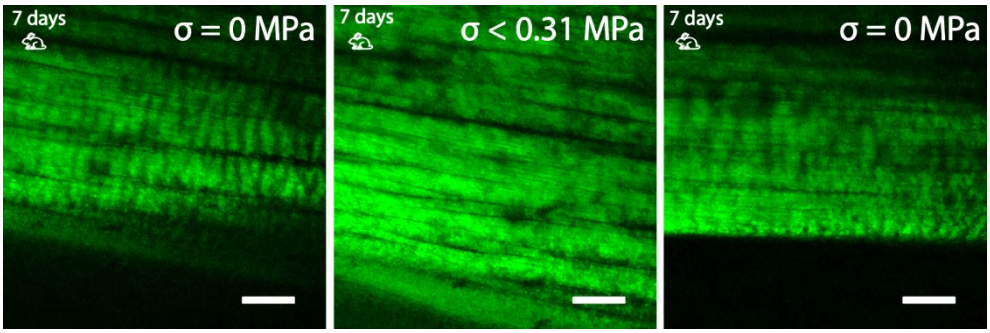


- Macro uniaxial tensile test: 4-month-old animal. (top) The tendon was sliding from the grips when performing the tensile test. The dark lines served as the markers for extensometry, and the red circles symbolize the spots detected by the automatic detection method. (bottom left) The engineering stress had a discontinuous character. (bottom right) The three methods used to detect the tendon elongation.

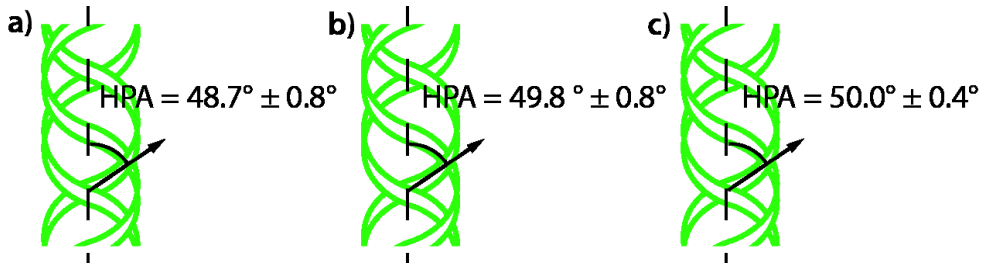


Results

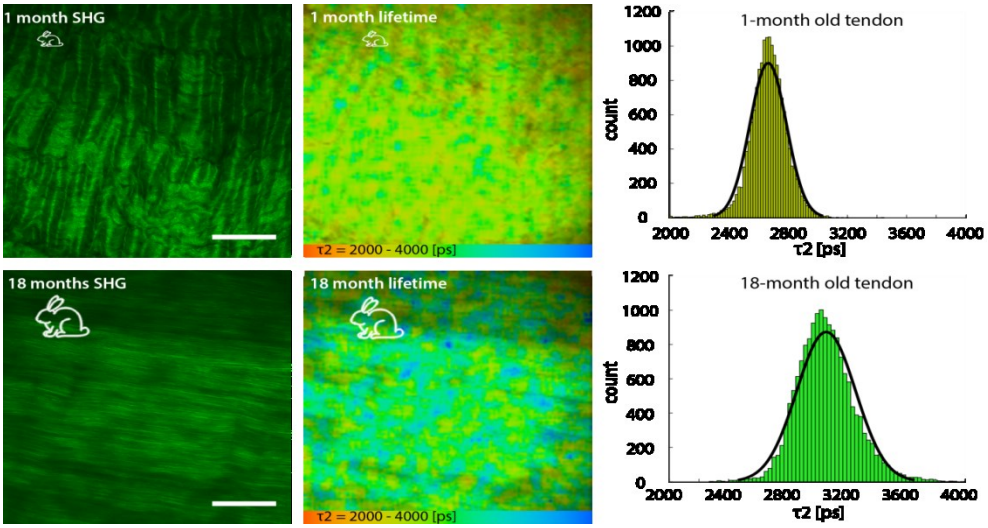
- The crimp pattern in the 7-day-old rabbit tendon disappeared before reaching minimum resolution of the force sensors.



- The PSHG microscopy at the load-free state revealed no significant difference in the HPA among the age groups.



- The lifetime images revealed the quality of the tissue micro-environment as the lifetime component τ_2 increased with age, and a significant difference was found between 1-month-old animals and 6-month-old, 18-month-old animals.



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Discussion, conclusion and additional information

4.1 General discussion and conclusion

The *in vitro* model was constructed to measure the production of type I collagen and visualise collagen fibres in the cultures with ascorbic acid and without acid. The aim was to label only the selected extracellular proteins and to preserve morphology of the cell culture. As immunostaining (IS) offers a wide pallet of labelling protocols[24,25], several modifications were implemented to meet this target. The modifications included placement of the cell culture on ice while applying the primary antibody and fixation of the cell culture after applying the primary antibody and washing. When comparing the cell culture with and without ascorbic acid (AA) treatment, the results showed higher cell growth and collagen deposition in culture with AA treatment, but no significant difference was found between expression levels of type I collagen gene. Besides, the cell culture was studied by label-free SHG microscopy. This measurement experimentally proved that the absence of AA in the cell culture, which directly affects the hydroxylation of proline, results in the loss of SHG signal. This validates the second-order susceptibility tensor model[26], in which hydroxyproline plays a crucial role while generating the second harmonic signal. As the presence of the SHG signal depends on the inner structure, it offers not only a quantitative but also qualitative tool for type I collagen investigation. This is the main advantage of non-linear label-free SHG microscopy over the classic IS methods when revealing pathological conditions[27] because IS scoring is often based on subjective expertise and returns results with high variability[28]. The advantages of SHG microscopy were even more pronounced when examining microtome section of tendon. The samples did not have to undergo multiple preparation steps[29,30] that affect morphology[31] and could be optically sectioned into 3-D label-free reconstruction. Thanks to the near infrared laser source, SHG microscopy also reduces scattering, photodamage and increases penetration depth of observation. The observation can be also directly enriched for the information about the orientation of collagen fibres. This approach relies on the fact that the SHG signal depends on the molecular orientation and incident beam polarization, respectively[32]. But sometimes, the sample cannot be scanned under different polarization angles, and therefore the fibre orientation must be retrieve from a single image. For that reason, the algorithm was developed to quantitatively determine the orientation regularity of collagen fibres. The results showed that there is a significant difference in the orientation regularity of fibres between the

microtome sections of the tendons from the young and old animals. The microtome sections from the young animals possessed high isotropy of fibres. On the other hand, the microtome sections from the old animals contained anisotropic fibres. The experiment on the intact tendons followed to investigate whether the differences of the orientation regularity had been caused by sectioning or by senescence. In this case, the load-free intact tendons had strong orientation regularity of the fibers within the main load-bearing axis. This fibrillar regularity was represented by a periodically undulating pattern – the crimp pattern. This pattern developed with age until it almost disappeared in the tendons from the old animals. The comparison of the results proved that the microtome sectioning modified the morphology of tendons, especially in the tendons from young animals.

Although there is not a generally accepted explanation for the cause and purpose of the crimp pattern, it is supposed that it reflects both the inner physiological and morphological status and provides an optimum mechanical response. The intact tendons were mechanically tested by the uniaxial tensile test but the data had high variability, and therefore, it was impossible to decide on the significance of the crimp pattern from the mechanical point of view. This problem was mainly caused by the tendon-grip interface, extensometry, and identification of the tendon geometry. For this reason, the tendon was stretched and simultaneously imaged by SHG microscopy. It was discovered that the crimp pattern had disappeared before reaching minimum resolution of the force sensors and reappeared immediately after returning to the initial elongation. This behaviour indicated a preload function of the crimp pattern which would lead to a lower increment of stress when activating muscles. After conducting the mechanical test with SHG microscopy, the visualization method was upgraded to polarized SHG (PSHG) microscopy. As the result, the helical pitch angle (HPA) of collagen molecule was detected for the intact tendons from animals of different age at the load-free state. This pixel resolution approach showed no change in the HPA in respect to age or mechanical loading. This finding supports the theoretical framework about the mechanical properties and stability of collagen molecule. On the other hand, fluorescence lifetime microscopy revealed significant changes in the micro-environment of tendon. These changes in fluorescence lifetime were probably caused by collagen cross-linkers which act as a reinforcement component that directly affect the mechanical properties of tendon.

Discussion, conclusion and additional information

4.2 Acknowledgement

I would like to thank to my promoters Assoc. Prof. Karel Jelen and Prof. Marcel Ameloot and co-promoters Dr. František Lopot and Prof. Virginie Bito for the critical mentoring. I also have to thank to my current leader, Dr. Jiří Janáček - department of Biomathematics, for supporting and mentoring me in the fields of signal processing and geometry. This work would not be possible without the support of The Charles University Grant Agency no. 956213 and the support by the Flemish government and Hasselt University.

The study on cell culture was approved by the Ethics Committee of the Institute for Clinical and Experimental Medicine and Thomayer Hospital in Prague, approval Nos. G 09-12-30, C.j. 2401/09, and G14-08-63, proved on 13th August 2014. The preparation of source pig tissue and cell isolation was primarily conducted by Dr. Elena Filova, prof. Jan Pirk, and Miroslav Konarik. The cell cultures, staining and quantification of collagen expression levels was conducted by Dr. Jana Liskova.

The microtome sections were prepared and stained in cooperation with Dr. Marketa Bacakova, Department of Biomaterials and Tissue Engineering, Institute of Physiology, the Czech Academy of Sciences, Videnska 1083, 14220 Prague, Czech Republic and with Dr. Eva Filova, Institute of Experimental Medicine, Department of Tissue Engineering, the Czech Academy of Sciences, Videnska 1083, 14220 Prague, Czech Republic. The orientation regularity analysis was completed in co-operation with Dr. Jiri Janacek, Department of Biomathematics, Institute of Physiology, the Czech Academy of Sciences, Videnska 1083, 14220 Prague, Czech Republic.

The PSHG microscopy was conducted in cooperation with Dr. Rik Paesen, UHasselt.

4.3 Curriculum vitae

Academic qualification

Master's degree **Optomechanics**
Department of Instrumentation and Control Engineering,
Faculty of Mechanical Engineering,
Czech Technical University, Prague, CR

Master's degree **Sport Sciences**
Faculty of Education and Sport,
Charles University, Prague, CR

Academic experience

Department of Biomathematics
Institute of Physiology,
Czech Academy of Sciences, Prague, CR

Department of Biomaterials and Tissue Engineering
Institute of Physiology,
Czech Academy of Sciences, Prague, CR

Department of Designing and Machine Components
Faculty of Mechanical Engineering,
Czech Technical University, Prague, CR

Selected publications

M. Bacakova, J. Pajorova, D. Stranska, **D. Hadraba**, et al., "Protein nanocoatings on synthetic polymeric nanofibrous membranes designed as carriers for skin cells," *International Journal of Nanomedicine* (accepted for publication in 12/2016).

M. Ostadal, J. Liskova, **D. Hadraba**, A. Eckhardt, "Possible Pathogenetic Mechanisms and New Therapeutic Approaches of Pes Equinovarus," *Physiological Research.* (accepted for publication in 12/2016).

J. Liskova, O. Babchenko, M. Varga, A. Kromka, **D. Hadraba**, et al., "Osteogenic cell differentiation on H-terminated and O-terminated nanocrystalline diamond films," *International Journal of Nanomedicine*, **10**, 869-84 (2015).

Discussion, conclusion and additional information

M. Bacakova, F. Lopot, **D. Hadraba**, et al., "Effects of fiber density and plasma modification of nanofibrous membranes on the adhesion and growth of HaCaT keratinocytes," *Journal of Biomaterials Applications* **29**(6), 837-53 (2015).

K. Novotna, M. Zajdlova, T. Suchy, **D. Hadraba**, et al., "Polylactide nanofibers with hydroxyapatite as growth substrates for osteoblast-like cells," *J Biomed Mater Res A.* **102**(11), 3918-30 (2014).

O. Fanta, **D. Hadraba**, F. Lopot, et al., "Pre-activation and muscle activity during frontal impact in relation to whiplash associated disorders," *Neuroendocrynology Letters* **34**(7), 708-16 (2013).

J. Vesely, **D. Hadraba**, H. Chlup, et al., "Constitutive Modelling and Histology of Vena Saphena," *Applied Mechanics and Materials* **465**, 249 - 254 (2013).

O. Fanta, J. Boucek, **D. Hadraba**, K. Jelen, "Influence of the front part of the vehicle and cyclist's sitting position on the severity of head injury in side collision," *Acta of Bioengineering and Biomechanics* **15**(1), 105-12 (2013).

D. Hadraba, "The effect of mechanical loading of hypokinesia on the structures of the extracellular matrix and functional changes in soft tissues," in K. Jelen, et al., *Biomechanical reflection of human hypokinetic stress and its identificators*, Karolinum, , Chapter 2.3 (2013), ISBN 9788024621821.

K. Jelen, F. Lopot, **D. Hadraba**, H. Herman, M. Lopotova, "The Women's Pelvic Floor Biomechanics," in: Injury and Skeletal Biomechanics, (2012). ISBN: 9789535106906.

K. Jelen, O. Fanta, R. Billich, **D. Hadraba**, P. Kubovy, "Whiplash Injury and Head Injury Criterion during Deceleration," *Transactions on Transport Sciences* **4**(4), 217-224 (2011).

Selected academic conferences

2016	**MECAHITECH**
	Bucharest, Romania
Oral presentation	*Design and realization of the instrument for measuring building material deformation in environmental chamber.*
2015	**Photonex, Nano-Spectroscopy & Bio-Imaging**
	Coventry, UK
Flash presentation	*Biomechanics and label-free imaging of connective tissue.*
2014	**18th International Microscopy Congress**
	Prague, CR
Poster presentation	*The response of nanofibrous PLA mats to mechanical stress.*
2013	**Focus on Microscopy**
	Maastricht, Netherlands
Oral presentation	*Label free optical methods for evaluation of mechanically exposed proteins in vein.*

Selected Grant Projects in Co-operation

956213	**Grant Agency of Charles University (main proposer)**
Topic	*The building proteins of the extracellular matrix detected by the microscopic and rheological methods.*
17-11898S	**Grant Agency of the Czech Republic**
Topic	*Nanosecond electric pulses for modulation of microtubule dynamics.*
P108/12/G108	**Grant Agency of the Czech Republic - Center of Excellence**
Topic	*Preparation, modification and characterization of materials by radiation.*

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