Acta Biomaterialia 37 (2016) 14–20

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Full length article

Lamellar and fibre bundle mechanics of the *annulus fibrosus* in bovine intervertebral disc

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ARTICLE INFO

Article history:
Received 3 December 2015
Received in revised form 29 March 2016
Accepted 5 April 2016
Available online 6 April 2016

Keywords:
Biomechanics
Intervertebral disc
Micromechanics
Second harmonic generation

ABSTRACT

The intervertebral disc is a multicomposite structure, with an outer fibrous ring, the annulus fibrosus, retaining a gel-like core, the nucleus pulposus. The disc presents complex mechanical behaviour, and it is of high importance for spine biomechanics. Advances in multiscale modelling and disc repair raised a need for new quantitative data on the finest details of annulus fibrosus mechanics. In this work we explored inter-lamella and inter-bundle behaviour of the outer annulus using micromechanical testing and second harmonic generation microscopy. Twenty-one intervertebral discs were dissected from cow tails; the nucleus and inner annulus were excised to leave a ring of outer annulus, which was tested in circumferential loading while imaging the tissue's collagen fibres network with sub-micron resolution. Custom software was developed to determine local tissue strains through image analysis. Inter-bundle linear and shear strains were 5.5 and 2.8 times higher than intra-bundle strains. Bundles tended to remain parallel while rotating under loading, with large slipping between them. Inter-lamella linear strain was almost 3 times the intra-lamella one, but no slipping was observed at the junction between lamellae. This study confirms that outer annulus straining is mainly due to bundles slipping and rotating. Further development of disc multiscale modelling and repair techniques should take into account this modular behaviour of the lamella, rather than considering it as a homogeneous fibre-reinforced matrix.

Statement of Significance

The intervertebral disc is an organ tucked between each couple of vertebrae in the spine. It is composed by an outer fibrous layer retaining a gel-like core. This organ undergoes severe and repeated loading during everyday life activities, since it is the compliant component that gives the spine its flexibility. Its properties are affected by pathologies such as disc degeneration, a major cause of back pain. In this article we explored the micromechanical behaviour of the disc's outer layer using second harmonic generation, a technique which allowed us to visualize, with unprecedented detail, how bundles of collagen fibres slide relative to each other when loaded. Our results will help further the development of new multiscale numerical models and repairing techniques.

1. Introduction

The intervertebral disc is the key element of spine flexibility; it resists high and diverse mechanical loadings while undergoing large and repeated strains. Disc mechanical behaviour is strongly dependent on its structure: the outer portion, the annulus fibrosus, is a strong ring of fibrous tissue that retains an inner core, the nucleus pulposus, which is gel-like in young, healthy animals and humans. The annulus is itself a composite structure formed of several lamellae, concentric layers containing fibres that are organised into bundles [1] and which are aligned within a lamella and at an angle between adjacent lamellae. When the spinal functional unit is subjected to physiological loading, annulus fibres can undergo high strains (up to 12%) without apparent damage [2–4].

The micromechanical behaviour of the disc is of particular interest to better understand the aetiology and progression of disc disorders [5]. For instance, disc damage such as herniation and rupture often initiates with delamination and/or the propagation of small cracks [6]. This is driving disc modelling towards multiscale approaches [7], using sophisticated methods to incorporate fine details such as the interaction between fibre bundles. This, in turn, has raised the need for experimental micromechanical data on

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http://dx.doi.org/10.1016/j.actbio.2016.04.002
1742-7061/

annulus ultrastructure. Such data are also required to inform the development of techniques of disc repair that aim at integrating a scaffold structure into the tissue [8,9]; indeed, cell survival and restoration of tissue strength depend on the mechanical compatibility between the engineered scaffold and the living tissue at the microscopic scale. A better knowledge of the latter could potentially help improve the design of these scaffolds.

The micromechanics of the whole disc has been investigated in some detail and a number of studies have focused on the mechanical properties of the annulus *in vitro*. For instance, uni- and bi-axial tests have been used to determine the stress-strain response of the annulus [10,11], down to the level of single lamella [12,13]. Most previous work has, however, inferred the mechanical behaviour of the tissue by tracking cells [14,15] or markers that were glued or photobleached on the tissue [10,14–16], or alternatively relied on relatively low resolution tissue tracking (for instance, 0.2 mm^2 elements [16,17]).

Second harmonic generation (SHG) is a multiphoton microscopic imaging technique that allows visualization of collagen network. It has been applied to investigate, for instance, cartilage [18,19], cornea [20], tendon and ligament [21,22] structure and mechanics, as well as structural disorder in intervertebral disc [23].

The aim of the present work was to describe and quantify the structural responses of the annulus subjected to micromechanical testing by means of SHG imaging, in order, particularly, to provide an insight on the mechanism of inter-bundle and inter-lamella straining.

2. Materials and methods

2.1. Sample preparation

Twenty-one intervertebral discs from cow tails were obtained from a local abattoir. Tails were frozen at −20 °C on the day of death and thawed overnight before testing (maximal frozen time: 5 months). Functional units were carefully dissected in order to expose the outer annulus fibrosus. The disc was detached from both adjoining endplates and its nucleus and inner annulus were excised to leave a ring of outer annulus of approximately 2 mm radial thickness (Fig. 1a).

2.2. Tensile testing

A custom built micro-straining rig adapted from [24] was utilized to test the samples (Fig. 1a). The rig consists of two micromanipulation stages, each carrying a flat hook 8 mm in height. The sample was mounted on the hooks and the stages were used to move the hooks apart, thus applying circumferential strain to the outer annulus. The horizontal macroscopic strain (ε) of the sample was measured from the displacement of the two stages, with 0.05 mm precision, as engineering strain: $\varepsilon = \varepsilon_{HM}^{eng} = 100 * (L - L_0)/L_0$, where L and L_0 are the initial and instantaneous distance between the inner faces of the flat hooks (Fig. 1), respectively. A load cell (LCM201 100N, Omega, Manchester, UK) was placed in series with one of the hooks.

First, a preload of 0.2 N was applied and strain was zeroed. Then the sample was strained in 1% strain steps until the region of interest (ROI) could not be imaged anymore (see below). The sample was kept moist during the test by surface application of phosphate-buffered saline.

2.3. Multiphoton imaging

The mechanical testing was performed under a confocal microscope (FluoView 300 and Olympus BX51) fitted with a 10x/0.4NA air objective (Olympus UPlanS Apo). The sample was illuminated with an 810-nm mode-locked femto-second Ti:Sapphire laser (Mira 900-D, Coherent Inc.) with a repetition rate of 76 MHz and a pulse width of 100 fs pumped by a 532 nm solid-state laser (Verdi V10, Coherent Inc.). This excites SHG in the sample, thus allowing visualization of collagen fibres (Fig. 1b–d). SHG was collected in the back-scattered direction using a photomultiplier (R3896 Hamamatsu) and the following combination of dichroic mirrors and filters to separate out the laser fundamental and any fluorescent signal; 670 nm long pass dichoric mirror (670dcxr Chroma), blue colour glass filter (CG-BG-39), narrow band pass filter (FF01-405/10 Semrock).

Samples were classed in three groups according to their apparent structure: Group L (Fig. 1b), for those ROIs where the fibres formed distinct lamellae, Group B (Fig. 1c), when the fibres were all parallel but formed bundles separated by a space in which no SHG was generated, and Group F (Fig. 1d), where the ROI showed a uniform array of fibres. In practice, group L (“lamellae”) represents those ROIs where the intersection between two lamellae was visible. In group B (“bundles”), only one lamella was visible, with aligned but clearly delimited and separated bundles of fibres. Group F (“fibres”) is similar to group B but the fibres were evenly distributes across the ROI, so that different bundles could not be clearly delimitated.

Images were acquired at each step of the mechanical test; the acquisition of an 800 × 600 pixels image (with sub-micron resolution) lasted about 30 s. When the ROI rotated out of the imaging plane and could not be imaged anymore, the test stopped.

2.4. Image processing and strain calculation

The centering of the ROI in the picture was improved after the test using ImageJ’s plugin for linear alignment (translation only) with scale invariant features transform [25]; this rigid translation does not affect the strain field.

Custom software was written in Matlab 2014b (The MathWorks, Inc., Natick MA) to obtain a displacement map from each series of images and calculate instantaneous microscopic strains. The first image of the series was divided into square elements which were automatically tracked in the following images by digital image correlation (Supplementary Content 1). The tracking of an element was considered unreliable if the correlation was lower than 0.5 (this values was chosen from preliminary tests); the size of these elements was set as small as possible to optimize reliability for each series of images (resulting between 12.8 and 28.8 μm^2). If the correlation was unreliable in more than 2% of the elements after adapting the element size, a 2D Wiener adaptive noise-removal filter was applied and the tracking run again. If the correlation was still unreliable in more than 2% of the elements, the image contrast was enhanced through histogram equalization. Finally, the remaining elements where the correlation failed (now less than 2%) were tracked by minimization of the squared difference between the nth and nth − 1 element grayscale values, thus obtaining correct tracking of all elements. The displacement map was obtained at each frame from the element’s displacement.

Displacements (u_x and u_y for the horizontal and vertical directions, respectively) were filtered with a local quadratic regression to reduce noise (span parameter = 0.5) and obtain smooth derivates to calculate local microscopic true strains. True linear (ε_x and ε_y) and shear (τ_{xy}) strain maps were calculated from the displacements at each frame in a coordinate system fixed with the image frame, i.e., x was aligned with the transverse plane and y with the vertical axis of the disc, as follows:

$$\begin{aligned} \varepsilon_x &= \ln(1 + \partial u_x/\partial x) \\ \varepsilon_y &= \ln(1 + \partial u_y/\partial y) \\ \tau_{xy} &= \ln(1 + \partial u_x/\partial y) + \ln(1 + \partial u_y/\partial x) \end{aligned} \quad (1)$$