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Development of a rapid matrix digestion technique for ultrastructural analysis of elastic fibers in the intervertebral disc

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ABSTRACT

Collagen and elastic fibers are two major fibrous constituents of the annulus fibrosus (AF) in the disc that contribute to its mechanical and viscoelastic properties. It was thought that elastic fibers play no substantial role in the function and properties of the disc as these fibers were irregularly distributed. Studies that have revealed highly organized elastic fibers with different regional orientation and distribution, while being strongly crosslinked with matrix, suggesting their contribution to disc structure-function properties. These studies that were performed by light microscopic analysis of histologically prepared samples, have not been able to reveal the fine-scale architectural details of the elastic fiber network. Since elastic fibers are intermingled with other fibrous components of the disc and mostly obscured by the extracellular matrix, it is difficult to demonstrate their ultra-structural organization using scanning electron microscopy (SEM).

Therefore the aim of this study was to develop a rapid matrix digestion technique for ultrastructural analysis of the disc elastic fibers. This study provides a new method for fundamental visualization of elastic fibers and their architecture in the disc. Through the ultra-structural analysis, the relationship between structure and function, as well as the role of elastic fibers on AF mechanical properties can be studied. This method may be used to develop a three-dimensional map of elastic fibers distribution within the disc, which would provide valuable information for designing tissue engineered scaffolds for AF repair and replacement.

1. Introduction

The major functional role of the intervertebral disc is to transfer loads in six degrees of freedom, while providing flexibility to the spinal column (Costi et al., 2007). To perform this dynamic behavior, the annulus fibrosus (AF) of the disc deforms with load application and then recovers to its original shape and size after load removal (O'Connell et al., 2011). The AF, with its multi-lamellar structure, is comprised of three fibrous elements; collagen, elastic fibers and micro-fibrils. Collagen fibers present as tape- or cord-shape that run a wavy course with a width of 1–20 μm, approximately. Micro-fibrils of diameter 30–100 nm are usually observed as a delicate meshwork. Elastic fibers are about 0.2–1.5 μm in diameter, and are generally twisted or straight strands that sometimes branch to form a course network (Ushiki, 2002).

Previously, elastic fibers have been suggested to play no substantial role in the mechanical properties and function of the AF, due to their irregular distribution (Yu, 2002). However, studies have reported that elastic fibers are highly organized in the AF and their distribution and orientation vary in different regions (Smith and Fazzalari, 2006, 2009). Also biochemical studies suggest that elastic fibers may be strongly cross-linked to the matrix and some other components of the AF (Guo et al., 2012). Therefore, it appears that the elastic fibers found in the AF play an important role in contributing to its structure-function properties (Smith et al., 2008).

According to the above studies it was found that the abundant elastic fibers, exposed by histological staining, are distributed throughout all AF regions with higher density between the lamellae (Tavakoli et al., 2016). Furthermore, the manner by which elastic fibers are anchored into the disc's endplate (perpendicular or oblique), have provided new insights into their function (Wade et al., 2012). Also, the distinction between the organizational appearance of elastic fibers most likely hinges on the functional requirement of elastic tissues, supporting the hypothesis that a high degree of organization of elastic fibers in different regions of the AF may indicate their contribution to mechanical properties (Green et al., 2014).

Recent studies have revealed that two distinct organizations of elastic fibers are present in the AF. In the lamella, elastic fibers align

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parallel to the collagen fibers, and are tightly packed within the surrounding matrix (Yu et al., 2007). The architecture of the network between two adjacent lamellae was reported to be dense, more complex and anisotropic compared to within lamellae (Tavakoli et al., 2016). These observations were acquired by light microscopy analysis of histologically prepared samples. While some of the techniques are advanced, none are able to provide the fine-scale architectural details of the elastic fiber network. On the other hand, since elastic fibers are intermingled with other fibrous components of the disc and mostly obscured by the extracellular matrix, it is difficult to demonstrate their ultra-structural organization using scanning electron microscopy (SEM). Therefore partial digestion of the matrix has been shown to be an effective technique for visualizing their architecture and ultra-structural organization at the ultra-scale (Crissman, 1986; Crissman and Guilford, 1984). It was previously described that some methods, including hot alkali and formic acid digestion that selectively remove non-elastin components (i.e. microfibrils, collagen, cellular elements and matrix), are effective for observing the architecture of elastin components in tissues by SEM (Ushiki, 2002). The SEM imaging of digested tissues for structural analysis is a well-described method that has been used to interpret vascular elastic fiber structure; however (Crissman and Pakulski, 1984), its application to the disc has not been studied.

Therefore the aim of this study was to develop a rapid matrix digestion technique for ultrastructural analysis of the disc elastic fibers, while preserving their structural integrity and organization. As the method will be employed for ultra-structural organization assessment of elastic fibers in the AF, three critical research key questions were addressed: 1) the effectiveness of the method on allowing visualization of elastic fibers, 2) the repeatability of the method and 3) its impact on structural alteration.

This study provides a new method for fundamental visualization of elastic fibers and their architecture. Through the ultra-structural analysis, the relationship between structure and function, as well as the role of elastic fibers on AF mechanical properties can be studied. This method may be used to develop a three-dimensional map of elastic fibers distribution within the disc, which would provide valuable information for designing tissue engineered scaffolds for AF repair and replacement.

2. Materials and methods

2.1. Sample preparation

Four lumbar sheep spines (18–26 months old) were obtained from a local abattoir, separated into bone-disc-bone segments with level L1/2 and stored at −30°C until used for analyses. Segments were then thawed overnight at room temperature while wrapped in saline soaked gauze. Each disc was dissected from the vertebral bodies. A 10 mm length of the anterior AF was separated from each disc (Fig. 1a) and was mounted with optimal cutting temperature compound (O.C.T, Tissue-Tek®) at an angle of approximately 30° to the transverse plane to identify the cutting plane (Fig. 1c) and stored at −30°C until used. As used previously, a cutting plane with an angle of about 30° was used to cut almost orthogonal to some lamellae and almost parallel to the others (Schollum et al., 2009). Four samples from adjacent sections (thickness 30 μm) were cut using a cryostat microtome (Leica Biosystems, CM3050) from each of four discs (Fig. 1d) and were mounted on poly-L-lysine coated microscope slides and stored at −30°C for further analysis. The first sample was not digested or treated (control sample). The second sample was used for histology and stained with orcein to visualize elastic fibers (orcein treated sample). The final two samples were digested in 0.5 M NaOH solution and sonicated for 15 min at 37°C (digested sample). One of these two samples was then soaked in water at 70 °C for 5 min to remove collagen fibers, leaving the elastic network (digested + heat treated sample). All three treated samples were then dried in a vacuum oven overnight (37°C and −80 kPa) and sputter coated with platinum at 2 nm thickness for SEM imaging.

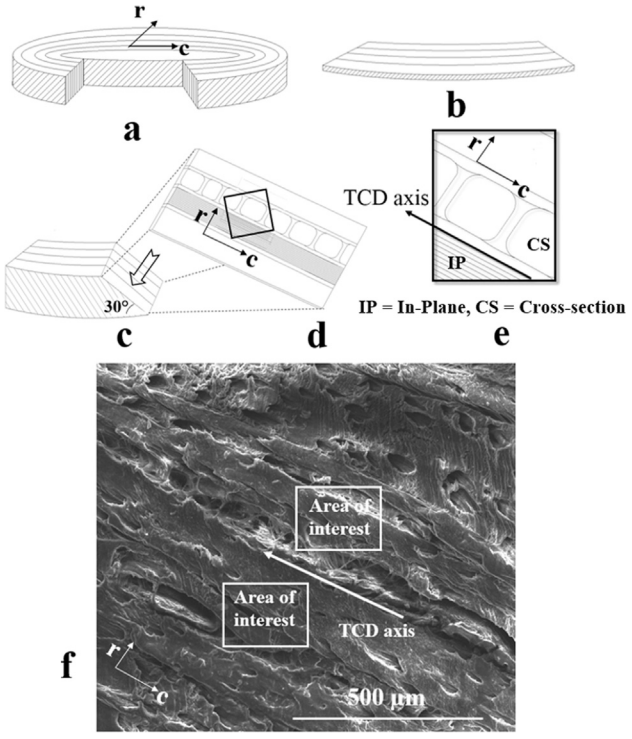

Fig. 1. Schematic drawing of sample preparation by frozen cutting of 30 μm thickness samples. (a) Anterior AF strips (10 mm in length) were separated from the disc, (b) Samples were cut by microtome to a thickness of 30μm along the cutting plane (approximately 30°), (c, d) Anterior AF strip and frozen section preparation at an angle of approximately 30° to the transverse plane. Scanning electron microscopy images were captured perpendicular to the samples' surface (z plane), as shown in (c), along the direction indicated by the arrow, (e) Schematic of two adjacent lamellae and their reference axis being tangential to the circumferential direction (TCD) for measuring fiber orientation, and (f) SEM images of a partially digested sample indicating the areas of interest and the TCD axis. "r and c" represent radial and circumferential directions respectively.

2.2. Orcein staining

Orcein staining was used to visualize the elastic fibers using a conventional histologic method (Yu et al., 2002). Briefly, the sample was soaked in orcein solution for 40 min after having been kept in ethanol (70%) for 5 min at room temperature. It was then soaked in tap water for 5 min. The dehydration process was accomplished by passing the sample through a series of increasing ethanol concentrations. The sample was soaked sequentially twice in 70%, and once each in 90% and 100% ethanol for approximately two minutes. The sample was then placed in a second 100% ethanol solution for 30 seconds to ensure that all water was removed. Finally, the sample was washed in xylene twice and mounted with DPX (mountant for histology). The presence and location of elastic fibers were visualized in the histologically prepared samples using light microscopy analysis (Brightfield BX50, Olympus, Japan).

2.3. Sample digestion and heat treatment

Partial NaOH digestion was performed in accordance with the method used on arteries to prepare the samples for SEM (Crissman and Pakulski, 1984). Briefly, dissected samples were placed in 0.5 M NaOH solution and sonicated (SONIC-950WT, MRC, Germany) for 25 min at 37 °C. The ultrasonic frequency was 25 KHz with a probe diameter of 3