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A method for visualization and isolation of elastic fibres in annulus fibrosus of the disc

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ABSTRACT

A simple and cost effective protocol for visualization and isolation of the elastic fibres network in the annulus fibrosus (AF) of the disc is explained, to provide other researchers a method that can be applied in disc ultrastructural analysis, biomechanical assessment of elastic fibre and tissue engineered scaffold fabrication. This protocol is developed based on simultaneous sonication and alkali digestion of tissue that eliminates all matrix constituents except for elastic fibres, which is applicable for different species including human. Thin samples harvested from ovine, bovine, porcine and human, which are commonly used in disc research, were exposed to 0.5 M sodium hydroxide solution along with sonication (25 kHz) in distilled water for defined periods of time at room temperature. Post heat treatment removed collagen fibres via the gelatinization process, for visualization of elastic fibres.

1. Introduction

The annulus fibrosus (AF) of the intervertebral disc, with its multi-lamellar structure is comprised of three fibrous elements; collagen, elastic fibres and micro-fibrils. Elastic fibres, as insoluble components of the extracellular matrix (ECM) in the AF, consist of a cross-linked elastin core surrounded with a template of fibrilin based micro-fibrils [1,2]. As reported for other connective tissues, elastic fibres are approximately 0.2–1.5 μm in diameter, and are generally twisted or straight strands that sometimes branch to form a course network [3]. Elastic fibres are distinguishable by histology staining using orcein [4] and resorcin-fuchsin [5] or using immunostaining [6] and can be visualized via light microscopy.

Early studies revealed an irregular distribution and low volume fraction of elastic fibres compared to other fibrous constituents [7], and suggested that elastic fibres play no substantial role in the mechanical properties, structure and function of the AF [8]. However, later studies have reported that elastic fibres were highly organized in the AF, their distribution varied in different regions and that they may interact with the ECM [4–6,8–11]. According to these studies it was found that abundant elastic fibres, visualized by histological staining, were distributed across the AF regions with higher density between AF lamellae (the inter-lamellar matrix, ILM). On the other hand, perpendicular or oblique anchoring of the AF elastic fibres into the disc endplates [12], as well as one study that revealed selective elimination of elastin fibres from the AF alters disc mechanical properties [13], have provided new insight into elastic fibres function in the disc.

The clinical relevance of elastic fibres is not fully understood. However, in contrast to healthy discs, where well organized and abundant elastic fibres exist, scoliotic discs have a sparse and disrupted elastic fibre network with a loss of lamellar structure [10]. This disorganization could be involved in the progression of spinal deformity and increase of disc metalloproteinase level [14]. Metalloproteinases can degrade elastin and release elastin peptides into the ECM [15]. Elastin peptides are able to trigger a series of biologic events, including an increase in cell calcium flux [16], metalloproteinase up-regulation [17], and cell proliferation [18]. Scoliosis discs, which are more vulnerable to degeneration compared to healthy ones, along with evidences interpreting an increase of irregularity of lamellae structure with increase of age, may show relation between elastic fibre network disorganization and disc degeneration [19].

From a biomechanical point of view, it is likely that elastic fibres play a role on the AF structural integrity via providing lower stiffness and absorbing more energy compared to the lamellae, indicating the biomechanical role of the ILM [20–22]. Our recent study identified that the well-organized and highly crosslinked elastic fibre network significantly contributes to both nonlinear elastic and failure mechanical properties of the ILM [23].

Understanding the elastic fibre ultrastructure-function relationship is a critical step towards understanding the clinical role of these fibres

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in disc herniation and degeneration, as well as for presenting potential therapeutic targets for preventing herniation and degeneration. Recent therapeutic treatments have been focused on regeneration of the ECM to support the function of the elastic fibre network [24,25]. To date, the mechanisms for elastogenesis are not well defined; however, it has been shown that different receptors, various growth factors and specific culture conditions are able to mediate the biosynthesis of elastin [26,27]. Using injectable scaffolds with the ability to sustain release of lysyl oxidase enzyme may lead to regeneration of elastic fibres via crosslinking of tropoelastin [28]. Fabricating new scaffolds that mimic disc ultrastructure increases the chance of regeneration in disc tissue engineering and AF replacement. The first step towards enhancing our knowledge about the role of elastic fibres is to visualize their ultrastructure. During our focused research on disc structure and biomechanical function, we found that there lacked a method to visualize the ultra-structural organization of elastic fibres in the disc. Histology and light microscopic-based studies are able to explain elastic fibre distribution, density and structure at the micro level across the AF, however, they can't be used for ultrastructure analysis [29]. Furthermore, enzymatic treatments are not only expensive but also hard to be optimized to limit the non-specific degradation of other AF constituents [13]. No other method for removing disc matrix and other components except elastin was reported; however, autoclaving, formic acid and sodium hydroxide digestion were reported to be used to elucidate the role of elastic fibres in different tissues, mainly heart valves and vessels [30–36]. Among the methods, alkali digestion has been reported to result in more purified elastin and leaving elastin inter-molecular crosslinks intact [37]. Ultrasound waves (sonication) during alkali digestion were used to expose networks of elastic fibres in vascular tissue for scanning electron microscopy observation [35]. This rapid method (compare to the hot alkali digestive technique) was shown to be effective in isolation and exposing intact networks of delicate elastic fibres in blood vessels which do not contain large amount of elastic tissues. Using the sonication alkali digestion method, it was shown that the surface appearance of elastic fibres, without a coat of microfibrils, were comparable to that reported for purified elastin fibres by other methods including alkali, autoclaving, chemical and enzymatic digestion [38–41]. Also it was confirmed that remaining fibres after sonication alkali digestion were elastic fibres [33,35,42].

Based on our research (published research and unpublished data), we have shown, for the first time that the sonication alkali digestion method can be optimized to expose the elastic fibre network in human, ovine, bovine and porcine AF of the disc [22,42]. We describe here a protocol for visualization and isolation of delicate network of elastic fibres across different regions of the AF. For visualization, partial digestion can be controlled to expose the elastic fibre network, which includes some level of remnant ECM. For isolation, full digestion can be performed to completely separate elastic fibres from the region of interest. This protocol has proved to be applicable to different species and different level of disc location that are widely used in disc research.

2. Materials and method

2.1. Materials

2.1.1. Disc tissue (annulus fibrosus)

- Human and animal (ovine, bovine or porcine) annulus fibrosus

2.1.2. Reagents

- Sodium hydroxide powder, reagent grade, 97% (Sigma Aldrich, cat. no. 655104)
- Distilled water
- Optimal cutting temperature compound (O.C.T., Tissue-Tek®, cat. no. 25608-930)
- Platinum

2.1.3. Equipment

- Magnet stirrer
- Freezer (−20 °C)
- Trimming blade with handle (Feather®, Type (s), cat. no. 130)
- Cryostat microtome
- Petri dishes with lid
- Pyrex Beaker with 150 mm diameter
- Ultrasonic processor with standard probe size 6 mm (MRC, sonic-950 watt)
- Water bath with temperature control
- Delicate tissue forceps (straight)
- Filter forceps (flat-tip forceps, bent) or delicate tweezer
- Surgical blade (size 11) with handle
- Micro-tube racks
- 100 ml medical syringe
- Water spray bottle
- Vacuum oven
- Aluminium stubs for Scanning Electron Microscope and double adhesive tape
- Spotter coater
- Scanning Electron Microscope (Inspect F50, FEI)

2.1.4. Reagent setup

2.1.4.1. Sodium hydroxide solution (digestion media). Mix 20 g sodium hydroxide powder in 1000 ml distilled water and stir for 10 min under 200 rpm and at room temperature using a magnet stirrer (to prepare 0.5 N sodium hydroxide solution in distilled water). Sodium hydroxide dissolves in distilled water easily and a clear solution is achieved. Solution can be stored for 2 weeks at 4 °C.

2.2. Method

2.2.1. Sample preparation for digestion

1- Remove fat and flesh from thawed spine using a surgical blade. Dissect disc tissues from vertebral bodies (upper and lower bones including endplates) using a trimming blade, spray with saline and store at −20 °C. While frozen, separate a tissue section of 10 mm length from region of interest (anterior, lateral, posterior, etc.) from disc tissue utilizing a surgical blade and mount with optimal cutting temperature compound at desired angle to the transverse plane to identify the cutting plane. Keep the mounted section in freezer (−20 °C) for at least 3 h. From the prepared section, cut adjacent samples (30 μm thickness) using a cryostat microtome. Using delicate forceps, collect thin samples from cryostat microtome and put them in a petri dish, gently spray the sample and close the lid immediately to avoid sample dehydration. Place sample in the freezer (−20 °C) as soon as possible.

CRITICAL STEP Do not collect the samples prepared from the first several cuts due to possible tears that might develop during cutting. Only collect intact samples with minimal structural deficiency. Collect and handle samples with care to avoid structural damage. Since damage to the samples may occur during each stage of this process, their careful handling is required and damaged samples should not be used for further analysis.

2- Fill a Pyrex beaker with 200 ml distilled water. Place the beaker into the water bath that is set to 70 °C. While temperature equilibrium is underway, continue the following procedure.
3- Place the sample at room temperature for 15 min before starting digestion. This step assists with gradual thawing of the sample and helps the samples to be easily detached from the petri dish.