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Tissue composition regulates distinct viscoelastic responses in auricular and articular cartilage

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ABSTRACT

It is well-accepted that articular (ART) cartilage composition and tissue architecture are intimately related to mechanical properties. On the other hand, very little information about other cartilage tissues is available, such as elastin-rich auricular (AUR) cartilage. While thorough investigation of ART cartilage has enhanced osteoarthritis research, ear cartilage reconstruction and tissue engineering (TE) could benefit in a similar way from in-depth analysis of AUR cartilage properties. This study aims to explore the constituent–function relationships of AUR cartilage, and how elastin influences mechanical behavior.

Stress–relaxation indentation and tensile tests were performed on bovine ART and AUR cartilage. Elastase incubation was performed to simultaneously deplete elastin and sulfated glycosaminoglycans (sGAG), while hyaluronidase incubation was used to deplete sGAG-only, in order to systematically investigate matrix components in material behavior.

ART and AUR cartilages showed different viscoelastic behaviors, with AUR cartilage exhibiting a more elastic behavior. Higher equilibrium properties and limited viscous dissipation of strain energy were observed in AUR cartilage, while ART cartilage exhibited a rapid viscous response and high resistance to instantaneous loading.

In conclusion, loss of sGAG had no effect on auricular mechanics in contrast to articular cartilage where GAG loss clearly correlated with mechanical properties. Auricular cartilage without elastin lost all compressive mechanical integrity, whereas in articular cartilage this was provided by collagen. This work shows for the first time the involvement of elastin in the mechanical behavior of ear cartilage. In future, this data can be used in AUR cartilage TE efforts to support reproduction of tissue-specific mechanical properties.

1. Introduction

Numerous publications have investigated articular (ART) cartilage, and have shown how composition and architecture relate to mechanics (Buschmann and Grodzinsky, 1995; Frank et al., 1987; Lai et al., 1991; Li et al., 2000; Little et al., 2011; McCutchen, 1982; Mow et al., 1990, 1980; Mow and Mansour, 1977; Spilker et al., 1992). ART cartilage is composed of water, proteoglycan aggregates (PGA), and dense type-II collagen (Mow and Lai, 1979). PGA are large macromolecules formed of a linear hyaluronate (HA) backbone onto which proteoglycan monomers (carrying sulfated glycosaminoglycan, sGAG) are attached (Mow et al., 1990; Mow and Lai, 1979). The carboxylated and sulfated GAGs are negatively charged at physiological pH, which leads PGA to seek their maximum solvent domain and become trapped in the collagen network (Mow and Lai, 1979). Immobilized PGA impede fluid flow through the tissue matrix (Comper et al., 1990) and contributes to mechanical integrity by resisting fluid efflux induced by tissue compression (Mow and Lai, 1979). A second contribution to tissue mechanics originates from fixed charged density (FCD) generated by charged groups on PGA, which increases concentration of mobile counter ions, maintaining electroneutrality and creating an intra-tissue osmotic swelling pressure (Buschmann and Grodzinsky, 1995). The specific composition of ART cartilage confers viscoelastic properties that distribute high contact loads during gait (McCutchen, 1982; Mow et al., 1980; Speirs et al., 2014).

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In contrast, very little information about ear, or auricular (AUR), cartilage is available. AUR cartilage is elastic cartilage (Ross et al., 1995), and contains an additional network of elastic fibers (Keith et al., 1977; Ross et al., 1995). These fibers consist of highly cross-linked tropoelastin monomers complexed with Ø10–12 nm microfibrils (composed of a complex array of macromolecules) (Mithieux and Weiss, 2005). Elastic fiber diameter can reach up to several micrometers (Daamen et al., 2007) compared to substantially smaller collagen fibrils (Ø30–200 nm) (Keene et al., 1995). Spontaneous recoil of elastin is of entropic origin (Urry et al., 2002), allowing elastic fibers to confer tensile resilience to tissues (Mithieux and Weiss, 2005). Similar to ART cartilage, the collagen network is mostly type-II collagen (Naumann et al., 2002) and the most abundant sGAG are chondroitin-4 and -6 sulfate (Bentley and Rokosova, 1970). In addition to being present in extracellular space between elastic fibers, HA and PGA are colocalized with elastic fibers (Baccarani-Contri et al., 1990; Wusteman and Gillard, 1977), and there is no zonal differentiation in AUR cartilage. These differences between ART and AUR cartilage suggest different mechanics, yet no comprehensive characterization of AUR cartilage is available in literature.

Developing ear implants with mechanical properties mimicking native tissue is an end goal of ear cartilage TE strategies (Naumann et al., 2002; Nimeskern et al., 2013, 2014; Xu et al., 2005; Zhou et al., 2011). However basic understanding of constituent–function relationships, that are critical for reconstruction strategies, is lacking (Nimeskern et al., 2014). The aim of this study is to elucidate constituent–function relationships of AUR cartilage, and investigate the mechanical role of elastin using an enzymatic depletion approach, with ART cartilage as control. Elastin depletion can be achieved with pancreatic elastase, although it will simultaneously deplete sGAG (Smith et al., 2008). Specific sGAG depletion can be achieved by cleaving the HA backbone of PGA with hyaluronidase (Bara et al., 2012). In this study we use elastase and hyaluronidase enzymatic treatments to selectively deplete both sGAG and elastin, and sGAG-only, in AUR and ART cartilage, and evaluate mechanical properties.

2. Material and methods

2.1. Sample harvesting

Three ears and three knee joints from young bovine (age 5–8 months) were obtained fresh at the local abattoir. As these animals were harvested for food purposes, no ethical permission was required. The dermis was removed from the ears followed by resection of perichondrium from the cartilage surface. Knees were opened to expose tibia plateau cartilage, from which full thickness samples were sliced. Plugs (Ø5 × ~2 mm) were punched from one auricle ($n=24$) and tibial plateau ($n=24$). Smaller cores were punched to obtain Ø3 mm cores with matching Ø5 mm outer rings. Outer rings were immediately frozen (−80 °C) for use as native controls (biochemistry).

From the remaining two ears and knees, 24 AUR and 24 ART cartilage strips (30 mm × 1 mm × ~2 mm), for tensile testing and biochemistry, were obtained using an in-house tissue slicer to control dimensions. Parallel cartilage strips from adjacent tissue were cut and frozen immediately (−80 °C) for use as native controls (biochemistry).

During preparation, samples were kept wet with phosphate buffered saline (PBS) supplemented with antibiotic/antimycotic solution (Gibco, CA, USA), PBS(A/A).

2.2. Enzymatic elastin and sGAG depletion

Unless indicated otherwise, all chemicals were purchased from Sigma Aldrich, MO, USA. Elastin was selectively degraded using a technique for intervertebral discs (Smith et al., 2008). Six AUR and 6 ART cores, and 6 AUR and 6 ART strips were incubated at 37 °C on a mechanical shaker at 850 RPM for 24 h in 3 U/mL porcine pancreatic elastase (E0258) containing 10 KIU/mL aprotinin (A3428) and 200 mM Tris (tris(hydroxymethyl)-aminomethane) with pH adjusted to 8.6 (elastase-treated group). To account for associated effects of sGAG depletion by elastase, a group of samples for depletion of sGAG-only (6 AUR and 6 ART cores and 6 AUR and 6 ART cartilage strips) was incubated at 37 °C on a mechanical shaker at 850 RPM for 24 h in a 50:50 mixture of phosphate buffer (300 mM sodium phosphate, pH 5.35) and hyaluronidase (8000 U/mL hyaluronidase, H3884, 20 mM sodium phosphate, 77 mM sodium chloride, 0.01% [w/v] bovine serum albumin, pH 7.0) yielding a 4000 U/mL hyaluronidase concentration (hyaluronidase-treated group). Enzyme-control groups were treated identically to their counterpart (elastase- or hyaluronidase-treated groups) but no enzymes were added; elastase-control and hyaluronidase-control groups, respectively ($n=6$ each). After 24 h incubation, samples were rinsed for 24 h at 4 °C in deionized water and equilibrated in PBS for 24 h at 4 °C.

2.3. Stress–relaxation indentation testing

Stress–relaxation indentation testing (Stok et al., 2010) was performed on each plug (elastase-treated group, hyaluronidase-treated group, and their respective controls) before and after enzymatic depletion. Briefly, samples were placed in close-fitting stainless steel cylindrical wells. Testing was performed with a materials testing machine (Zwick Z005, Ulm, Germany) equipped with a 10 N load cell, built-in displacement control, and cylindrical, plane-ended, stainless steel indenter (Ø0.9 mm), and samples were immersed in PBS(A/A). A preload of 3 mN was applied to a sample to locate the surface and measure thickness, and held for 5 min. Four successive strain steps were applied in 5% strain increments of thickness, and specimens were left to relax for 25 min at each step. Hold time was defined as the time necessary to reach equilibrium. Measurements of instantaneous modulus (E^i_{in}), equilibrium modulus (E^i_{eq}) were determined for every sample (superscript "*i*" for indentation). Additionally, relaxation half-life time ($t^i_{1/2}$) was defined as the time required for stress to fall from its maximum (immediately after strain application) half-way to equilibrium (Nimeskern et al., 2013). Tests were performed at room temperature.

2.4. Tensile testing

Tensile testing was performed on each cartilage strip (elastase-treated group, hyaluronidase-treated group, and their respective controls) before and after enzymatic depletion. Samples were tested in tension using the protocol of Fessel et al. (2014). Briefly, sample extremities (10 mm) were wrapped in tissue paper (Kimtech Science Precision Wipes, Kimberly-Clark Healthcare, GA, USA) fixed with cyanoacrylate glue (Loctite 454, Düsseldorf, Germany) to prevent slippage. Cross-sectional area was measured with an in-house laser telemetric device (Fessel et al., 2014). Mechanical testing was performed with a material testing machine (Zwick 1456) equipped with a 200 N load cell and built-in displacement control. A 0.1 MPa preload was applied, samples were preconditioned for 15 cycles from 0% to 11% strain before elongation in four successive strain steps of 5%, 7%, 9% and 11%, and left to relax for 120 s between each step. Tensile equilibrium modulus, E^t_{eq}, was computed from equilibrium stress measured for each step. Tests were performed at room temperature.

2.5. Biochemical analysis

Following testing sample wet weight was determined, and samples were freeze-dried and weighed again to determine water content. Biochemical analysis was performed on native control samples, enzyme-treated samples, and their respective controls. Samples were digested overnight at 60 °C in papain solution of 50 mM $Na_2H_2PO_4$, 5 mM ethylenediaminetetraacetic acid, 2 mM dithiothreitol, 1 mg/mL papain (P4762) at pH 6.8. A 1,9-Dimethylmethylene Blue (pH 3.0) assay gave sGAG content (Farndale et al., 1986), monitored by absorbance at 520 nm using an Infinite F200 PRO plate reader (Tecan, Männedorf, Switzerland). Shark chondroitin sulfate (C4384) was used as standard, and sGAG content was normalized to sample dry mass, sGAG/dm (μg/mg).

Elastin content was measured using the Fastin™ Elastin Assay (Biocolor, Carrickfergus, UK). Samples were converted to water soluble α-elastin by 7 h heat extraction cycle at 100 °C in 0.25 M oxalic acid before adding the kit's dye. Absorbance was measured at 513 nm, and α-elastin from bovine neck ligament (provided by manufacturer) was used as standard. Elastin content was normalized to sample dry mass, ELN/dm (μg/mg).

Hydroxyproline content was measured to estimate collagen using the Total Collagen Assay (QuickZyme Biosciences, Leiden, NL), as described previously (Prockop and Udenfriend, 1960).

2.6. Histology

Additional native control samples and incubated samples were embedded in Tissue-Tek OCT medium (Sakura, Alphen aan den Rijn, NL), quenched in cold ethanol (−80 °C) and sectioned at 5 μm. Sections were stained with Safranin-O (84120) with light green solution (62110) as counterstain for sGAG. A 1:9 picric acid/Sirius Red solution (80456 and Direct Red 80, ABCR, Karlsruhe, Germany, respectively) was used for collagen visualization. For elastin visualization,