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In vitro glycation of articular cartilage alters the biomechanical response of chondrocytes in a depth-dependent manner

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SUMMARY

Objective: To determine if increasing cartilage cross-links through *in vitro* glycation of cartilage explants can alter the biomechanical response of chondrocytes to compressive deformation.
Method: Bovine osteochondral explants were either incubated with cell culture solution supplemented with ($n = 7$) or without ($n = 7$) ribose for 42 h in order to induce glycation. Deformation-induced changes in cell volume, dimensions and local tissue strains were determined through confocal laser scanning microscopy (CLSM) and the use of a custom built micro-compression device. Osteochondral explants were also utilized to demonstrate changes in depth-wise tissue properties, biomechanical tissue properties and cross-links such as pentosidine (Pent), hydroxylysyl pyridinoline (HP) and lysyl pyridinoline (LP).
Results: The ribose treated osteochondral samples experienced reduced cell volume deformation in the upper tissue zone by ~8% ($P = 0.005$), as compared the control samples, through restricting cell expansion. In the deeper tissue zone, cell volume deformation was increased by ~12% ($P < 0.001$) via the transmission of mechanical signals further into the tissue depth. Biomechanical testing of the ribose treated osteochondral samples demonstrated an increase in the equilibrium and dynamic strain dependent moduli ($P < 0.001$ and $P = 0.008$, respectively). The biochemical analysis revealed an increase in Pent cross-links ($P < 0.001$). Depth-wise tissue property analyses revealed increased levels of carbohydrate content, greater levels of fixed charge density and an increased carbohydrate to protein ratio from 6 to 16%, 55–100% and 72–79% of the normalized tissue thickness (from the surface), respectively, in the ribose-treated group ($P < 0.05$).
Conclusion: In vitro glycation alters the biomechanical response of chondrocytes in cartilage differently in upper and deeper zones, offering possible insights into how aging could alter cell deformation behavior in cartilage.

Introduction

Like other connective tissues within the body, articular cartilage (AC) is susceptible to age-related changes. Nearly half of all persons over 65 years suffer from osteoarthritis (OA) in the USA[1]. Hence, it is not surprising that aging is a major risk factor in the development of OA[2]. The traditional explanation offered is that mechanical loading of AC over its life cumulatively affects it, resulting in "wear and tear" and eventual breakdown[3] of this joint tissue. However, recent research is suggesting that this is not the case[3–7].

Researchers are now focusing on understanding how cells respond to and convert mechanical signals into biomolecules aimed at tissue maintenance/remodelling[6–8]. Because the biological responses (i.e., cell metabolism) of chondrocytes (cells) in AC are related to the mechanical signals that they experience[6–9] understanding how mechanical signals can drive cell responses is important in understanding the aging process and how it can trigger the development of OA. Cartilage research has so far demonstrated that aging increases the stiffness of both the ECM (the extracellular matrix of AC)[10–15] and chondrocytes[16,17], decreases the viscoelastic properties of the local cellular matrix (termed the pericellular matrix or PCM)[17], modifies cellular metabolism and metabolic pathways[3–7,11,18–20] and ultimately increases the tissue's susceptibility to fracture[13–15]. Age-related deterioration of AC appears to be a problem that involves both tissue and cellular-scale changes. Thus, understanding how cells respond to

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deformation within aged AC is important in identifying mechano-biological differences due to tissue aging.

It is well documented that advanced glycation end products (AGEs) accumulate within the ECM[12–15,17–20] during aging, resulting in increased levels of cross-linking, particularly in long-lived proteins, such as collagens. Treatment of AC with sugars, such as ribose[11,14,15,19,20] and threose[13,19–21], have been used to mimic the aging process to demonstrate that cross-linking can alter the mechanical properties of AC and possibly cause tissue dysfunction, degeneration and/or OA[3–9,13–15]. Although these treatments provide a faster means of inducing *in vitro* glycation, they may not completely mimic the age-related non-enzymatic products that form *in vivo*[22–25].

Other studies have investigated the biomechanical response of chondrocytes and/or their PCM in AC by utilizing unconfined compression[26,27] and indentation techniques[28–30]combined with confocal microscopy or by modeling the stress/strain fields within AC[31–33]. These studies have provided a better understanding of how the ECM/PCM can contribute towards modifying the response of chondrocytes to compression. However, it is not known how or if inducing cross-links within AC can alter the response of chondrocytes to deformation in cartilage.

This study's aim is to investigate how chondrocytes in normal and artificially cross-linked osteochondral explants mechanically respond to deformation. In order to achieve this, ribose was used to induce cross-linking within AC. Chondrocyte deformation was investigated through the use of a custom built micro-compression device and confocal laser scanning microscopy (CLSM). Osteochondral explants were also utilized to demonstrate changes in biomechanical tissue properties. Biochemical analysis for cross-links such as pentosidine (Pent), hydroxylysyl pyridinoline (HP) and lysyl pyridinoline (LP) were performed through high-performance liquid chromatography (HPLC)[21]. This enabled us to chemically confirm changes in the levels of cross-links between untreated (control) and ribose-treated (treated) groups. Digital densitometry (DD), Fourier Transform infrared spectroscopic imaging (FTIRI) and polarized light microscopy (PLM) were also performed to investigate depth-wise structure and composition of the tissues.

Methods

A brief description of the materials and methods is given here. More details are listed in the Supplemental Material.

Sample preparation

Surface intact osteochondral plugs (ø = 31 mm) were harvested from the lateral aspect of the distal pole of patellae obtained from seven separate bovine animals. The patellae were obtained from a local abattoir (Atria Oyj, Kuopio, Finland) and the age of the animals at the time of slaughter was 18 ± 1 months. The total number of animals utilized in this study was based on previous glycation research that demonstrated statistical differences from utilizing patellar cartilage from a total of seven bovine animals[21]. Each plug had two osteochondral samples removed (ø = 8 mm; Fig. 1) and these were randomly placed into a control or treated group ($n = 7$/group). Samples were then incubated (for 42 h) in cell culture medium with 30 mM of ribose supplemented in the medium (treated group) or without (control group) ribose supplementation. The remnant tissue portions (Fig. 1) were carried through the identical incubation process described for the osteochondral samples. After incubation, each osteochondral sample had a smaller osteochondral plug (ø = 2.9 mm) removed for biomechanical testing (Fig. 1). The remaining osteochondral samples were cut into

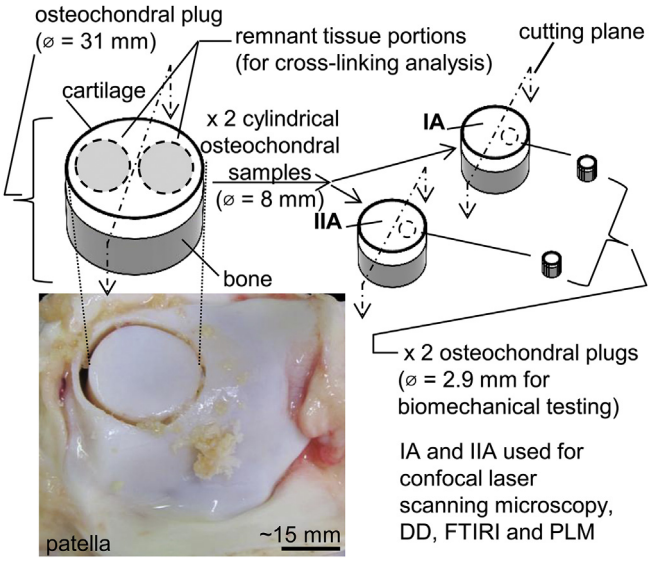

Fig. 1. Schematic diagram illustrating the sample preparation steps used for the cartilage samples.

hemi-cylindrical samples and were used for cell deformation experiments (Fig. 1) and then subsequent analysis for depth-wise tissue structure and composition. The cartilage from the remnant tissue portions was removed from underlying bone; had their wet weights determined and analyzed biochemically for cross-links.

Cell deformation experiments

The hemi-cylindrical samples were stained for 30 min with both calcein-AM (5 μM, Invitrogen, Eugene, OR, USA) and propidium iodide (60 μM, Sigma, Ronkonkoma, NY, USA) for live and dead cells, respectively. Following staining, samples were positioned cut tissue side down (with the exposed bone surface against the loading bar) into a sample bath of a custom built stainless steel micro-compression device [Fig. 2(a)-i]. The samples were kept hydrated by filling the chamber with Dulbecco's Modified Eagle Medium (DMEM) (1 g/l low glucose, Gibco, Eugene, OR, USA) throughout testing.

An inverted CLSM Zeiss LSM 700 (Zeiss Axio Observer .Z1, Carl Zeiss, Oberkochen, Germany) was used for microscopic imaging [Fig. 2(a)-ii]. To perform cell deformation experiments, the micro-compression device was fitted into the microscope stage [Fig. 2(a)-ii]. Initial contact between the articular surface and the side of the sample bath was visually confirmed through the use of a 10× magnification objective. Tissue thickness was then estimated by measuring the distance between the surface to cartilage–bone interface through the confocal microscope computer software (Zen 2009, Carl Zeiss, Germany). An undeformed image stack of the entire sample (from the tissue surface to cartilage–bone interface) and higher magnification image stacks of chondrocytes within the "upper", "middle" and "deep" tissue zones were taken prior to deformation (using a ×10 magnification objective and an ×40 magnification oil immersion objective, respectively; Fig. 2(b), (c)). Each sample was then deformed by 15% and the tissue was allowed to relax for 45 min[21]. After tissue deformation, image stacks of chondrocytes within the "upper", "middle" and "deep" tissue zones and fully deformed tissue thickness image stacks were obtained [see Fig. 2(b), (c)]. For the purposes of this study, the upper zone was defined to be from the cartilage surface up to 15% depth from the surface, the middle zone from 15% to 41% depth from the tissue