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Micromechanical properties of canine femoral articular cartilage following multiple freeze-thaw cycles

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ABSTRACT

Tissue material properties are crucial to understanding their mechanical function, both in healthy and diseased states. However, in certain circumstances logistical limitations can prevent testing on fresh samples necessitating one or more freeze-thaw cycles. To date, the nature and extent to which the material properties of articular cartilage are altered by repetitive freezing have not been explored. Therefore, the aim of this study is to quantify how articular cartilage mechanical properties, measured by nanoindentation, are affected by multiple freeze-thaw cycles. Canine cartilage plugs (n = 11) from medial and lateral femoral condyles were submerged in phosphate buffered saline, stored at 3–5 °C and tested using nanoindentation within 12 h. Samples were then frozen at −20 °C and later thawed at 3–5 °C for 3 h before material properties were re-tested and samples re-frozen under the same conditions. This process was repeated for all 11 samples over three freeze-thaw cycles. Overall mean and standard deviation of shear storage modulus decreased from 1.76 ± 0.78 to 1.21 ± 0.77 MPa (p = 0.91), shear loss modulus from 0.42 ± 0.19 to 0.39 ± 0.17 MPa (p=0.70) and elastic modulus from 5.13 ± 2.28 to 3.52 ± 2.24 MPa (p = 0.20) between fresh and three freeze-thaw cycles respectively. The loss factor increased from 0.31 ± 0.38 to 0.71 ± 1.40 (p = 0.18) between fresh and three freeze-thaw cycles. Inter-sample variability spanned as much as 10.47 MPa across freezing cycles and this high-level of biological variability across samples likely explains why overall mean "whole-joint" trends do not reach statistical significance across the storage conditions tested. As a result multiple freeze-thaw cycles cannot be explicitly or statistically linked to mechanical changes within the cartilage. However, the changes in material properties observed herein may be sufficient in magnitude to impact on a variety of clinical and scientific studies of cartilage, and should be considered when planning experimental protocols.

1. Introduction

Articular cartilage is a viscoelastic heterogeneous material divided into layered zones with varying material properties and functionalities (Silver et al., 2002). The extracellular matrix (ECM) is heterogeneous in nature, where variations exist in composition, structure and vascularity at a micro-level. It is composed of proteoglycans, collagens and glycoproteins, which are all macromolecular components (Silver et al., 2002). Cartilage also contains chondrocytes that become embedded within the matrix, maturing and dividing to deposit new cartilage. Its primary function is to maintain a smooth surface allowing lubricated frictionless movement and to help transmit articular forces, therefore minimising stress concentrations across the joint (Nigg and Herzog, 2006).

Knowledge of material properties of cartilage is crucial to understanding its mechanical function and morpho-functional alterations that occur during ageing, disease and injury (Wen et al., 2012, Kleemann et al., 2005). Whilst valuable data in isolation, material property information is also crucial to other mechanical analyses, including computational models that attempt to predict *in vivo* joint

Abbreviations: AFM, Atomic Force Microscopy; CSM, Continued Stiffness Measurement; E, Elastic Modulus; ECM, Extra Cellular Matrix; G',, Shear Modulus; G", Storage Modulus; PBS, Phosphate Buffered Solution; SD, Standard Deviation, Standard Error Mean (SEM)

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behaviour (e.g. Wang et al., 2014, Guo et al., 2009, Pena et al., 2006). Material properties of articular cartilage ECM have been widely reported utilising varying testing, storage and preservation techniques (e.g. Shepherd and Seedhom, 1997, Kleemann et al., 2005, Wen et al., 2012). Specific testing techniques have changed over time and varied according to investigator preference and overall experimental goals. In general, however, all studies seeking to quantify the mechanical behaviour of biological tissues strive to maintain biological fidelity of the testing conditions in the experiment; for example testing fresh tissue samples under hydrated conditions that are representative of the internal environment of the studied organism (Brandt et al., 2010). However, accomplishing this may be challenging for numerous reasons including the need for transportation between dissection and testing locations, availability or failure of testing equipment and the desire to test large sample numbers from individual specimens thereby minimising tissue waste. In such circumstances it is standard practice to store and preserve samples, often requiring tissue to undergo one or more freeze-thaw cycles before mechanical tests can be carried out (e.g. Wilusz et al., 2013, Lau et al., 2008; Li et al., 2006).

Therefore in situations where logistical limitations prevent testing of fresh samples, it is beneficial to explore if preservation of tissues samples through freezing can be utilised without compromising mechanical properties. In recent years there have been a number of systematic investigations into the effects of multiple freeze-thaw cycles on the mechanical properties of ligaments and tendon (Huang et al., 2011, Moon et al., 2006, Woo et al., 1986). Although some variation between individual studies exists, these analyses suggest that ligament and tendon tissue can undergo a minimum of two freeze-thaw cycles before significant changes to their material properties occur, thereby providing important constraints on experimental designs involving these tissues. However, despite its fundamental importance to joint biomechanics, to the best of our knowledge, no such data exists exploring the effect of more than one freeze-thaw cycle on material properties of articular cartilage. The aim of this paper is therefore to quantify how articular cartilage mechanical properties are affected by multiple freeze-thaw cycles directly addressing this important gap in knowledge. Dynamic nanoindentation is used to determine the shear storage modulus (G'), shear loss modulus (G''), elastic modulus (E) and the loss factor ($tan\ \delta$) of canine femoral condyle articular cartilage across three freeze-thaw cycles.

2. Materials and methods

2.1. Specimen preparation

One disease free canine cadaveric knee joint from a skeletally mature Staffordshire Bull cross mix was dissected 36 h after being euthanized. Ethical permission for use of this cadaveric material was granted by the Veterinary Research Ethics Committee, University of Liverpool (VREC327). Healthy articular cartilage samples (n = 11) measuring < 1 cm^2, were harvested from the medial and lateral bilateral femoral condyles (Fig. 1) using a low speed band saw (deSoutter Medical, Bucks, UK). Gross examination of the samples showed no sign of fibrillation or wear.

Following dissection, each of the 11 samples were submerged in phosphate buffered saline (PBS) and stored in cooled temperatures (3–5 °C) for up to 12 h until they were tested when still fresh using nanoindentation techniques, as detailed below. Following testing, all 11 samples were then frozen at −20 °C for up to 48 hours. Samples were then individually thawed for three hours at 3–5 °C and re-tested using the same nanoindentation protocol after having undergone one freeze-thaw cycle. This was completed within one hour and hydration of cartilage was maintained through constant exposure to PBS prior to and during testing (Brandt et al., 2010). This freeze-thaw procedure was repeated for three cycles and material properties of all 11 samples were measured after each freeze-thaw cycle. Samples were specifically thawed in cooled conditions (3–5 °C), as room temperatures have been shown to thaw cartilage samples too quickly and cause damage to the ECM (Szarko et al., 2010).

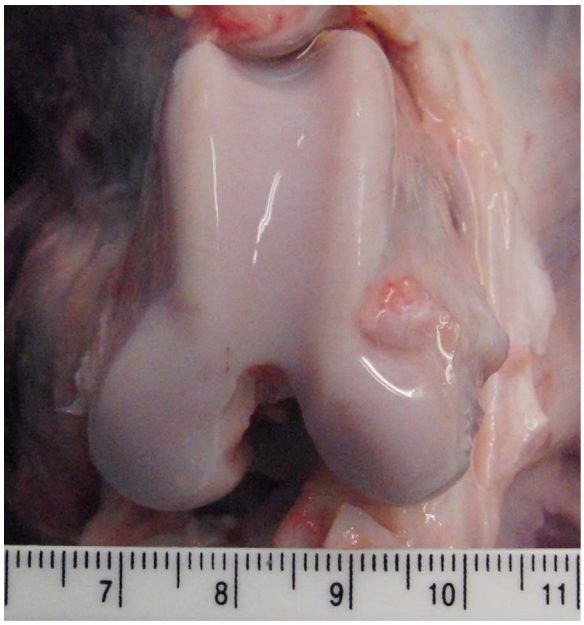

Fig. 1. Photograph of the medial and lateral femoral condyle of the canine specimen to scale (cm), from which samples were harvested.

2.2. Nanoindentation testing

Cartilage samples underwent dynamic nanoindentation (G200 Nanoindenter, Keysight Technologies, Chandler, AZ, USA) equipped with an ultra-low load DCM-II actuator utilising a Continuous Stiffness Measurement (CSM) module to determine the micromechanical complex shear modulus.

Samples were mounted into a custom made liquid cell holder, with a 1 cm radius and 2 mm deep well, which could allow partial submersion of the samples in PBS during testing (Fig. 2). Samples were then examined under the built-in optical microscope to randomly select ten indent locations per sample (> 100 μm spacing between each indentation to avoid immediate overlap) totalling 110 measurements per cycle of freezing. Given that it was not possible to differentiate between microstructural features in the cartilage with the optical microscope, indentation sites were based on topographical homogeneity for accu-

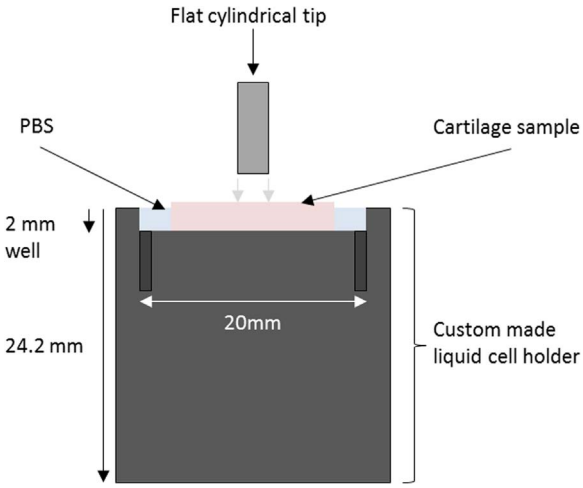

Fig. 2. A schematic of the custom made liquid cell holder holding the cartilage sample and phosphate buffered saline (PBS).