Ultrasound in Med. & Biol., Vol. 40, No. 9, pp. 2162–2171, 2014
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0301-5629/$ - see front matter

http://dx.doi.org/10.1016/j.ultrasmedbio.2014.03.016

- *Original Contribution*

COLLAGEN AND CHONDROCYTE CONCENTRATIONS CONTROL ULTRASOUND SCATTERING IN AGAROSE SCAFFOLDS

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(*Received* 18 *November* 2013; *revised* 19 *February* 2014; *in final form* 12 *March* 2014)

Abstract—Ultrasound imaging has been proposed for diagnostics of osteoarthritis and cartilage injuries *in vivo*. However, the specific contribution of chondrocytes and collagen to ultrasound scattering in articular cartilage has not been systematically studied. We investigated the role of these tissue structures by measuring ultrasound scattering in agarose scaffolds with varying collagen and chondrocyte concentrations. Ultrasound catheters with center frequencies of 9 MHz (7.1–11.0 MHz, −6 dB) and 40 MHz (30.1–45.3 MHz, −6 dB) were applied using an intravascular ultrasound device. Ultrasound backscattering quantified in a region of interest starting right below sample surface differed significantly ($p < 0.05$) with the concentrations of collagen and chondrocytes. An ultrasound frequency of 40 MHz, as compared with 9 MHz, was more sensitive to variations in collagen and chondrocyte concentrations. The present findings may improve diagnostic interpretation of arthroscopic ultrasound imaging and provide information necessary for development of models describing ultrasound propagation within cartilage. (E-mail: satu.inkinen@uef.fi) **© 2014 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Ultrasound, Imaging, Articular cartilage, Scattering, Attenuation, Chondrocyte, Collagen, Agarose.**

INTRODUCTION

Articular cartilage covers the ends of articulating bones. The unique mechanical characteristics of cartilage facilitate the load-bearing function of a joint (Mow et al. 1992). Articular cartilage consists of a fluid phase, a solid phase and cells, that is, chondrocytes. The solid phase consists mainly of collagens and proteoglycans. The collagen network is responsible for the tensile and dynamic properties of cartilage (Basser et al. 1998; Maroudas et al. 1973; Mow et al. 1992). Type II collagen is the main component of the solid matrix. The concentration of collagen in articular cartilage can be as high as 200 mg/mL (Basser et al. 1998; Mow et al. 1992). Collagen fibers form a highly organized network within cartilage (Mow et al. 1992; Muir et al. 1970). In osteoarthritis (OA) collagen degradation occurs, causing changes in the orientation and concentration of the network (Eyre 2002). Chondrocytes are responsible for maintaining the cartilage tissue by secreting and synthesizing collagens, proteoglycans, hyaluronan and non-collagenous proteins into the extracellular matrix (Buckwalter and Mankin 1998; Mow et al. 1992). Chondrocytes occupy only 1%–5% of the total volume of cartilage; the mean chondrocyte density in human cartilage is around 14 million cells/mL (Mow et al. 1992; Stockwell 1978). Chondrocytes are ellipsoid in shape and have a diameter around 10 μm (Stockwell 1978). However, their size, orientation and concentration change with the depth of the cartilage (Hunziker et al. 2002; Stockwell 1967). Importantly, chondrocyte concentration varies with the progression of OA. Hypercellularity and cell cloning are typical of early OA, whereas hypocellularity can be seen in advanced OA (Goldring and Marcu 2009; Mankin and Lippiello 1970).

Quantitative ultrasound arthroscopy has been proposed as a tool in the diagnosis of cartilage injuries and osteoarthritis *in vivo* (Kaleva et al. 2011; Virén et al. 2011). It has been reported that quantitative ultrasound measurements can detect mechanical degradation of superficial cartilage (Saarakkala et al. 2004, 2006) and

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enzymatic digestion of superficial collagens (Saarakkala et al. 2004; Saïed et al. 1997; Töyräs et al. 1999). In early OA, the superficial collagenous layer degenerates, causing fibrillation. Fibrillation of the cartilage surface can be detected using an intravascular ultrasound device (Virén et al. 2009). Fibrillation has been reported to increase the ultrasound roughness index and to reduce the ultrasound reflection coefficient (R) at the articular surface (Virén et al. 2009). It has been proposed that the decrease in ultrasound backscattering from internal cartilage structures that occurs in OA arises from changes in the structure of the collagen network (Chérin et al. 1998). In addition, apparent integrated backscattering (AIB) increases within surgically or spontaneously repaired cartilage, implying that the structure of the collagen network is abnormal in the regenerated tissue (Virén et al. 2010, 2012a). These findings are in line with early reports on collagen as the main scatterer of ultrasound within soft tissue, for example, myocardium (O'Donnell et al. 1981).

Chondrocyte concentration is low in articular cartilage compared with other soft tissues, and the contribution of chondrocytes as ultrasound scatterers at clinical frequencies is still unclear. It has been proposed that chondrocytes could affect high-frequency ultrasound backscattering in cartilage (Männicke et al. 2014). In addition, the size of a chondrocyte is optimal for Rayleigh scattering at high ultrasound frequencies, and chondrocytes can be detected at frequencies ≥100 MHz (Leicht and Raum 2008).

Despite continuous efforts to develop ultrasound methods for characterization of articular cartilage, the contributions of separate structural components of the cartilage to ultrasound backscattering have not been systematically studied. In this study, we used a clinical intravascular ultrasound device operating at frequencies of 9 and 40 MHz to evaluate ultrasound scattering within agarose scaffolds with varying collagen and chondrocyte concentrations. Agarose gel scaffolds have been used in studies involving ultrasound measurements because their water content is similar to that of soft tissues (Kohles et al. 2012). Using agarose, we produced a highly standardized, well-controlled test system and hypothesized that collagen significantly affects ultrasound scattering at clinical frequencies and that backscattering from chondrocytes is minimal.

METHODS

Sample preparation

Nine sets of agarose gel (4%) scaffolds containing type I collagen were prepared. Each set included seven samples, and each sample within a set had a different concentration of collagen (0, 1.5625, 3.125, 6.25, 12.5, 25 and 50 mg/mL). Similarly, nine sets of seven scaffolds, each containing a different chondrocyte concentration (0, 1, 2, 4, 8, 16 and 32 million cells/mL) were prepared. Additionally, to reach collagen concentration closer to those of human articular cartilage, nine separate samples with a collagen type I concentration of 100 mg/mL were prepared. All series included a reference agarose (4%) sample without collagen or chondrocytes.

For the collagen series, 100 mg of type I collagen (calf skin, Sigma-Aldrich, St. Louis, MO, USA) was first dissolved and mixed in 1 mL of 0.1 M HCl. This solution was diluted to 8% low-melting agarose gel at 40°C temperature (NuSieve GTG agarose low melting, Cambrex Bio Science, Rockland, ME, USA). We thereby obtained collagen concentrations of 50, 25, 12.5, 6.25, 3.125, 1.5625 and 0 mg/mL, and the agarose concentration was 4%. For the collagen concentration of 100 mg/mL, first 200 mg/mL type I collagen was dissolved in 1 mL of 0.1 M HCl and carefully diluted to 8% agarose so that the final collagen concentration was 100 mg/mL and the agarose concentration was 4%.

For the chondrocyte series, cartilage was first collected from bovine femoral condyles. Cartilage was cut into small pieces and placed in a digestion medium overnight. Chondrocytes were isolated as described previously (Qu et al. 2006), except that hyaluronidase digestion was omitted and we used a high-glucose Dulbecco's modified Eagle medium (DMEM) (Lonza, Verviers, Belgium) instead of low-glucose DMEM. Chondrocytes and medium were filtered through a 115-μm nylon filter and washed twice with phosphate-buffered saline (PBS) after digestion. Chondrocytes were mixed in 5 mL of DMEM chondrocyte culture medium (Qu et al. 2012). The cells were counted in a Bürker chamber. First, the cell suspension was diluted to a ratio of 1:10 in PBS and then pipetted into the Bürker chamber. The cells were counted from three lattices, and the three counts were averaged. Finally, the dilution series of 64 million, 32 million, 16 million, 8 million, 4 million, 2 million and 0 chondrocytes per milliliter were made. Low-melting agarose was prepared at 40°C, and chondrocytes were mixed in agarose (8%) to obtain the final agarose content of 4%. Great care was taken during the mixing process to prevent air bubbles from forming within the sample.

Finally, liquid gel suspensions were pipetted into cylindrical wells (diameter = 5.9 mm, height = 3.9 mm) on a sample plate. A glass sheet was pressed against the surface of the plate, thereby ensuring a flat surface on top of the wells during the gelation process.

Ultrasound imaging

All samples were imaged with an intravascular ultrasound device (Clear View Ultra, Boston Scientific, San Jose, CA, USA) (Fig. 1). Two catheters with center

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