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● *Original Contribution*

ULTRASOUND BIOMICROSCOPY IMAGING FOR MONITORING PROGRESSIVE TRYPSIN DIGESTION AND INHIBITION IN ARTICULAR CARTILAGE

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Abstract—This study reports an ultrasound biomicroscopy (UBM) imaging approach to monitor the progressive trypsin-induced depletion of proteoglycan (PG) and its inhibition in articular cartilage. Three fresh, normal bovine patellae were obtained and four full-thickness cartilage-bone specimens were prepared from the lower medial side of each patella. One sample was used as a control and the other three were divided into three groups: Groups A, B and C (n = 3 for each group). After a 40 min 0.25% trypsin digestion, samples from group A were continuously digested in trypsin solution, while those in groups B and C were immersed in physiologic saline and fetal bovine serum (FBS), respectively, for another 280 min. The trypsin penetration front was observed by UBM and M-mode images were acquired using 50 MHz focused ultrasound and custom-developed software. The results show that the 40 min trypsin digestion degraded nearly the whole surface layer of the cartilage tissue. Further digestion in trypsin or residual digestion in saline for 280 min depleted most of the PG content, as observed in groups A and B. The replacement of trypsin with a physiologic saline solution only slightly slowed the digestion process (group B), while trypsin inhibitors in FBS stopped the digestion in approximately 1.5 h (group C). The normalized digestion fractions of the digested tissues were calculated from ultrasound data and histology sections, and then compared between the groups. Without the use of FBS, 80% to 100% of the full thickness was digested, while this number was only approximately 50% when using FBS. Our findings indicate that the UBM imaging system could provide two-dimensional (2-D) visual information for monitoring progressive trypsin-induced PG depletion in articular cartilage. The system also potentially offers a useful tool for preparing cartilage degeneration models with precisely controlled PG depletion. (E-mail: ypzheng@ieee.org) © 2009 World Federation for Ultrasound in Medicine & Biology.

Key Words: **Articular cartilage, Ultrasound, High frequency ultrasound, Ultrasound biomicroscopy, Proteoglycan depletion, Trypsin digestion, Trypsin inhibitors, Osteoarthritis.**

INTRODUCTION

Articular cartilage (artC) is a low frictional, load-bearing soft tissue that provides almost frictionless support for several joints. The material composition of artC is a multiphase hydrated mixture mainly composed of 5%–10% proteoglycan (PG), 10%–20% collagen, and 60%–80% water (Mow et al. 2005). Aggregating PGs are negatively charged bio-macromolecules that are enmeshed in the collagen matrix (Lai et al. 1991; Maroudas 1976). Therefore, PGs play an important role in determining the electrochemical and mechanical properties of articular cartilage, such as shear modulus (Zhu et al. 1993), compressive modulus (Qin et al. 2002; Zheng et al. 2001, 2005), swelling strain (Narmoneva et al. 1999; Wang and Zheng 2006) and swelling aggregate modulus (Flahiff et al. 2004; Narmoneva et al. 2002; Wang et al. 2007, 2008a). It has been widely reported that the loss of PGs is one of the earliest signs of osteoarthritis (OA), which is one of the most common joint diseases. Advanced stage OA is characterized by a partial or total loss of cartilage tissue and the direct contact of bone across the joint (Armstrong and Mow 1982; Martini 2004; Sandy 2003; Torzilli et al. 1990). Early diagnosis of artC degeneration is very important for the treatment of OA. However, conventional methods using X-ray imaging can only detect very late stage OA. Most existing magnetic resonance imaging (MRI) systems do not have sufficient resolution for the assessment of artC. Conventional ultrasound scanners are not suited to the direct study of OA, as their resolution is also usually insufficient to image changes in artC.

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Further, it is difficult to scan the whole articulating surface with an ultrasound probe located outside the body. New approaches using arthroscopic imaging can provide more information about artC degeneration processes such as surface fibrillation but it remains challenging to assess early stage OA using video inspection. To evaluate new therapies such as mosaic plasty, cultured chondrocyte transplantation and even the use of tissue-engineered cartilage, it is necessary to develop sensitive assays of the therapeutic outcome (Detterline et al. 2005; Fu et al. 2003). During the last decade, various techniques have been developed for this purpose, including mechanical indentation (Toyras et al. 2001), quantitative ultrasound (Saied et al. 1997; Joiner et al. 2001; Hattori et al. 2003; Nieminen et al. 2002,), ultrasound combined with indentation (Zheng and Mak 1996; Suh et al. 2001; Laasanen et al. 2002), water-jet indentation (Lu et al. 2009), compression (Zheng et al. 2004a), osmotic loading (Wang et al. 2008a), optical coherence tomography (OCT) (Herrmann et al. 1999), air-jet OCT indentation (Huang et al. 2009) and electromechanical measurement (Legare et al. 2002).

Various *in vitro* and *in vivo* animal models mimicking human articular cartilage degeneration have been reported in the literature. These models are intended to aid the development of new diagnostic approaches and the investigation of the mechanism of degeneration. To study how the loss of PGs results in changes in morphologic, biomechanical, acoustic and bioelectrical parameters, many studies have treated normal articular cartilage with enzymes such as trypsin. Such treatment depletes PGs, mimicking natural cartilage degeneration (Basser et al. 1998; Brown et al. 2007; Deng et al. 2007; Disilvestro and Suh, 2002; Harris et al. 1972; Hunziker and Rosenberg 1996; Laasanen et al. 2002; Moody et al. 2006; Nieminen et al. 2002; Qin et al. 2002; Saarakkala et al. 2004; Suh et al. 2001; Toyras et al. 2002; Zheng et al. 2001, 2004b). In terms of the understanding of how the loss of PGs affects different physical parameters of cartilage, tremendous achievements have been made using these trypsin-treated animal and human cartilage models. In spite of these advances, one fundamental problem in preparing these models has not been solved: how to quantify the degree of PG depletion during the trypsin treatment. In other words, how can we prepare a cartilage degeneration model with exactly the required degree of PG depletion?

The conventional way to quantify PGs in cartilage is to use histologic assays by binding different stains, such as safranin-O, toluidine blue, hematoxylin and eosin (H&E) and alcian blue, to differentiate different compositions in cartilage (Lyons et al. 2006). However, this method is time-consuming and invasive since it involves specimen collection, fixing, staining, slicing, imaging and analyzing. In many studies, pilot tests are used to determine the degree of PG depletion under a certain concentration of trypsin, operating temperature and period of treatment. Since variations in the cartilage thickness and in the distribution of PGs and chondrocytes are commonly observed between different specimens (Moody et al. 2006), it is difficult to control the trypsin digestion process using histologic techniques to create calibration standards. An alternative method is to conduct histologic examination after completing all necessary (nondestructive) tests on the trypsin-treated cartilage specimen, so as to quantify the PG depletion level of each specimen. This is time-consuming and, in many cases, the histologically examined PG depletion level may not be the level expected for the experimental design. Unfortunately, at that point, it is too late to alter the experiment based on this knowledge. Therefore, it is very important to have a real-time monitoring approach for preparing cartilage degeneration models with a defined PG depletion level. Furthermore, enzyme inhibitors such as fetal bovine serum (FBS) are commonly used to stop trypsin digestion at a certain PG depletion level (Xiang et al. 2006). Using routinely available methods, it is not possible to determine precisely how long it takes for FBS to stop the trypsin digestion process or how the inhibition process proceeds, although it has been estimated in previous studies that an inhibition solution of several inhibitor chemicals can stop trypsin digestion within approximately 1 h (Qin et al. 2002; Rieppo et al. 2003). In addition, although washing with saline after trypsin treatment is a very commonly used technique in the literature, we do not know whether replacing the trypsin solution with a saline solution can stop the digestion process.

Inspired by the reduction of elasticity of articular cartilage with depletion of PGs, a novel technique using high frequency ultrasound to monitor the process of trypsin digestion has been recently reported (Toyras et al. 2002; Nieminen et al. 2002; Zheng et al. 2004b; Wang et al. 2008b). The fundamental principle of this technique is that the reduction in the elasticity of PG-depleted cartilage leads to a decrease in its acoustic impedance and, thus, creates an artificial acoustic interface between the digested and undigested cartilage tissues. An ultrasound echo can be generated at this interface and this echo shifts as the trypsin digestion progresses deeper into the tissue. With continuous recording of ultrasound signals, the trace of digestion can be viewed in an M-mode ultrasound image. This novel technique allows real-time monitoring of the process of PG depletion induced by trypsin digestion, although the reported works are still very preliminary. These earlier studies were mainly focused on the feasibility of this technique (Toyras et al. 2002; Nieminen et al. 2002; Zheng et al. 2004b). The digestion speed of a certain concentration of trypsin in cartilage was recently reported (Wang et al. 2008b). In these studies, the ultrasound beam was focused at a single location in the articular cartilage. Thus, it is difficult to get an accurate digestion profile