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● *Original Contribution*

ACOUSTIC IMPEDANCE ANALYSIS WITH HIGH-FREQUENCY ULTRASOUND FOR IDENTIFICATION OF FATTY ACID SPECIES IN THE LIVER

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Abstract—Acoustic properties of free fatty acids present in the liver were studied as a possible basis for non-invasive ultrasonic diagnosis of non-alcoholic steatohepatitis. Acoustic impedance was measured for the following types of tissue samples: Four pathologic types of mouse liver, five kinds of FFAs in solvent and five kinds of FFAs in cultured Huh-7 cells. A transducer with an 80-MHz center frequency was incorporated into a scanning acoustic microscopy system. Acoustic impedance was calculated from the amplitude of the signal reflected from the specimen surface. The Kruskal–Wallis test revealed statistically significant differences ($p < 0.01$) in acoustic impedance not only among pathologic types, but also among the FFAs in solvent and in cultured Huh-7 cells. These results suggest that each of the FFAs, especially palmitate, oleate and palmitoleate acid, can be distinguished from each other, regardless of whether they were in solution or absorbed by cells. (E-mail: k_ito@chiba-u.jp) © **2016 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Acoustic impedance, Scanning acoustic microscopy, Non-alcoholic steatohepatitis, Free fatty acid, Liver, Lipid droplet.**

INTRODUCTION

With the high prevalence of metabolic disorders, the occurrence of non-alcoholic fatty liver disease (NAFLD) is increasing worldwide and emerging as an important social problem (Browning et al. 2004; Chalasani et al. 2012). In the United States, an estimated one-third of the population has NAFLD, and approximately 2% to 5 % of Americans also have non-alcoholic steatohepatitis (NASH) in addition to simple steatosis. NASH is a serious condition that can progress to cirrhosis and hepatocellular carcinoma (Vernon et al. 2011). Therefore, accurate discrimination of NASH from simple steatosis is a critical issue in current clinical practice.

Currently, ultrasound imaging is widely used for diagnosing liver diseases (Bouchard et al. 2014; Mottin et al. 2004; Palmentieri et al. 2006) because it is non-invasive, non-ionizing and low in cost compared with other medical imaging modalities. However, the gold standard for diagnosis of NASH remains liver biopsy (Burt et al. 1998; Cortez-Pinto et al. 2006) because distinguishing NASH from simple steatosis using B-mode ultrasound imaging is difficult (Saadeh et al. 2002). Nevertheless, liver biopsies have severe drawbacks; they carry the risks of side effects and a high rate of false-negative determinations caused by sampling errors (Tobkes and Nord 1995). Therefore, alternative methods to diagnose NASH are urgently needed in clinical practice.

For more than three decades, many approaches to the quantitative characterization and diagnosis of liver diseases using ultrasound have been investigated (King et al. 1985; Lizzi, 1983; Ophir, 1991). For example, ultrasound elastography seems to be one of the most promising methods (Cassinotto et al. 2014; Castera et al. 2008; Doherty et al. 2013; Nightingale et al. 2015; Sporea et al. 2012). Recent studies have reported that shear wave velocity correlates with serum-marker test results and fibrosis stage (Fierbinteanu Braticevici et al. 2013; Kang et al. 2015; Yoneda et al. 2010) and has the potential to distinguish NASH from other liver diseases. For example, Kang et al. (2015) reported that shear wave elastography can accurately classify fibrosis stage in NAFLD rat models. However, the shear wave velocity method also has severe limitations; in particular,

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the results are unreliable in obese patients and in patients with ascites, jaundice or congestive liver failure (Kudo et al. 2013).

Another set of ultrasonic tissue characterization methods based on backscatter ultrasound spectrum analysis (Feleppa et al. 1996; Mamou et al. 2011; Oelze et al. 2004) and envelope statistics appear promising (Higuchi et al. 2014; Igarashi et al. 2010, 2011; Nakajima et al. 2010; Ricci et al. 2013; Yamaguchi and Hachiya 2010). With these methods, quantitative tissue parameters related to tissue microstructure are obtained from backscattered echo signal data. Transverse wave velocity, effective scatterer diameter or effective acoustic concentration can be estimated by these methods, and they can be related to acoustic properties of tissue, such as speed of sound, attenuation and acoustic impedance at the microscopic level (Bamber and Hill 1981; Fields and Dunn 1973; Hachiya et al. 1994; Lizzi et al. 1987). Nevertheless, no ultrasound technique has been entirely successful in quantitatively diagnosing NASH, although some reports successfully diagnose NAFLD (Acharya et al. 2015; Lin et al. 2014). Lin et al. (2014) successfully classified the patients with and without NAFLD based on the ultrasound backscatter coefficient. However, it remains challenging to distinguish NASH from simple steatosis.

The precise mechanisms of NASH progression are not fully understood. Several mechanisms, such as insulin resistance, endoplasmic reticulum stress, inflammation and oxidative stress, have been hypothesized to explain how free fatty acids (FFAs) may be important in NASH progression (Cortez-Pinto et al. 2006; Farrell and Larter 2006). Recent studies have indicated differences in FFA content between controls and patients with NASH (Allard et al. 2008; Puri et al. 2009). These differences are probably caused by an imbalance in dietary intake and/or impaired metabolism (Donnelly et al. 2005). Therefore, as a first step toward ultrasonic characterization of NASH, FFAs were investigated because they play an important role in NASH progression.

Some methods, such as gas chromatography and high-pressure liquid chromatography, were established to characterize FFAs (Borch, 1975; Fisk et al. 2014; Metcalfe and Schmitz 1961). These methods are precise and sensitive, but expensive and time consuming, even if the sample is small. Therefore, characterization of FFAs with high-frequency ultrasound was investigated. For differential diagnosis of NASH, the aim is to characterize acoustic properties of cells in the presence of various FFAs. The hypothesis is that if significant differences in acoustic properties exist among various types of FFAs, then an ultrasound-based approach could assess NASH with high reliability.

The long-term goal of this project is to develop a quantitative, non-invasive, *in vivo*, ultrasound-based approach for diagnosing NASH. At clinical frequencies in the range 5 to 15 MHz that are used to assess livers *in vivo*, ultrasound echo signals are dominated by scattering. This is due to the fact that both cell organization and tissue microstructure are smaller than the ultrasound wavelength. Ultrasound scattering originates from the variations in the spatial distributions of acoustic properties, such as mass density and compressibility (Insana and Brown 1993). In soft tissues, ultrasound backscattering can be fully explained by the spatial distribution of acoustic impedance (Insana and Brown 1993). Therefore, scanning acoustic microscopy (SAM) was selected for this initial study because it is capable of measuring acoustic impedance, as well as other tissue properties, at a microscopic level without making physical contact (Tanaka et al. 1984).

Recent studies have reported the ability of SAM to provide reliable estimates of acoustic properties of tissue components with a spatial resolution less than 20 μm. For example, there have been successful investigations of cell properties and cell thickness profiles (Kundu et al. 2000; Weiss et al. 2007), liver (Irie et al. 2016), vascular tissue (Saijo et al. 2004; Yamaoka et al. 2013), gastric cancer (Saijo et al. 1991), brain tissue (Hozumi et al. 2004; Saijo et al. 2007), ocular tissue (Radhakrishnan et al. 2013; Rohrbach et al. 2015) and lymph nodes (Miura et al. 2013). SAM can be used to measure numerous acoustic parameters such as speed of sound, attenuation and acoustic impedance, but novel methods used to measure only acoustic impedance with SAM are advantageous because they can be performed on living cells, fresh tissues and liquids (Fadhel et al. 2015; Hildebrand and Rugar 1984; Strohm et al. 2010; Yoshida et al. 2012). Therefore, SAM could be an ideal tool to investigate the influence of FFAs on liver cells.

The present study was aimed at elucidating the significance of acoustic impedance (Z) measurements using a SAM system in relation to FFA content in liver cells. This may determine how FFA content influences the acoustic properties of liver cells at a microscopic level. Three sets of experiments were performed using SAM with an 80-MHz center frequency transducer to measure acoustic impedance. The first set of experiments measured four pathologic types of mouse liver: untreated livers (control) and liver models of simple steatosis, NASH and cirrhosis. The acoustic impedance of a mixture of FFAs as “lipid droplets” was to be obtained in this experiment. The second set of experiments investigated five typical FFAs: palmitate, oleate, palmitoleate, linoleate and α-linolenic acid to determine their physical properties in isolation. The third set of experiments placed the same FFAs in a cultured cell solution. This