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● *Original Contribution*

CYTOMECHANICAL PERTURBATIONS DURING LOW-INTENSITY ULTRASOUND PULSING

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Abstract—To establish the therapeutic potential of low-intensity ultrasound, it is important to characterize its biophysical interactions with living cells. Here, through a series of single-cell direct observations, we show that low-intensity ultrasound pulsing would give rise to a dynamic course of cytomechanical perturbations at both the membrane and nucleus levels. Our investigation was conducted using a composite platform that coupled a 1-MHz ultrasound exposure hardware to a confocal microscopy system. Short ultrasound pulses (5 cycles, 2-kHz pulse repetition frequency) with a spatial-peak time-averaged intensity of 0.24 W/cm^2 (0.85-MPa peak positive acoustic pressure) were delivered over a 10-min period to adherent Neuro-2a neuroblastoma cells, and live imaging of cellular dynamics was performed before, during and after the exposure period. Bright-field imaging results revealed progressive shrinkage of cellular cross-sectional area (25%–45%, N = 7) during low-intensity ultrasound pulsing; the initial rate of size decrease was estimated to be 8%–14% per minute. This shrinkage was found to be transient, as the sonicated cells had recovered (at a rate of size increase of 0.4%–0.9% per minute) to their pre-exposure size within 30 min after the end of exposure. Three-dimensional confocal imaging results further revealed that (i) ultrasound-induced membrane contraction was volumetric in nature (21%–45% reduction), and (ii) a concomitant decrease in nucleus volume was evident (12%–25% reduction). Together, these findings indicate that low-intensity ultrasound pulsing, if applied on the order of minutes, would reversibly perturb the physical and subcellular structures of living cells. (E-mail: alfred.yu@hku.hk) © **2014 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Low-intensity ultrasound, Wave-cell interaction, Cytomechanics, Membrane, Nucleus, Real-time imaging.**

INTRODUCTION

At low intensity, the pulsing of ultrasound on living matter is well regarded as biologically tolerable in that thermal elevations would not be instigated unless gas nuclei were present to trigger acoustic cavitation (Miller et al. 2012). This dosimetric principle has long been the operating premise for diagnostic ultrasound imaging that essentially works *via* pulse-echo sensing. On the other hand, it is known that low-intensity ultrasound can also exert mechanical pressure on matter (Dalecki 2004), likely in ways similar to direct mechanical loading (Perry et al. 2009) or fluidic shear stress (McCormick et al. 2006; Saini et al. 2011). Consequently, non-thermal bio-effects may be elicited in living cells during sustained periods of ultrasound exposure (on the order of minutes), and these effects may be of therapeutic value (Pounder and Harrison 2008). Studies have indeed suggested that low-intensity ultrasound may accelerate fracture healing (Cheung et al. 2011; Claes and Willie 2007; Romano et al. 2009), foster cellular growth (Choi et al. 2011; Hill et al. 2005; Inubushi et al. 2008; Zhang et al. 2003) and modulate neuronal activity (King et al. 2013; Tufail et al. 2011). Nevertheless, current evidence on the treatment efficacy of low-intensity ultrasound is often considered to be controversial, as the extent of therapeutic effects varies greatly (de Albornoz et al. 2011; Zacherl et al. 2009).

To avoid prematurely asserting the therapeutic value of low-intensity ultrasound without an in-depth scientific understanding, it is imperative to establish the mechanistic details on how ultrasound-induced stimulatory bio-effects come about (ter Haar 2007). One particular aspect that needs to be properly characterized is the

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sequence of cytomechanical events involved in the process, especially because the wave-matter interactions instigated by low-intensity ultrasound are predominantly mechanical in nature (Dalecki 2004). However, only fragmented findings have so far been reported on this topic. A general trend that has been observed is post-exposure cytoskeletal remodeling (Hauser et al. 2009; Mizrahi et al. 2012; Noriega et al. 2013; Zhang et al. 2012), and it may result in the alteration of the cell's adherence characteristics (Mahoney et al. 2009; Roper et al. 2012; Zhou et al. 2004). In line with this trend, post-exposure bio-assays have revealed that a cell's mechanotransduction pathway would be activated by low-intensity ultrasound (Louw et al. 2013; Whitney et al. 2012), and in turn, its associated molecular signaling cascades would be modulated (Favaro-Pipi et al. 2010; Ito et al. 2012; Lu et al. 2009). Although ultrasound-induced alterations in downstream cellular functions have been confirmed by these studies, it remains unknown as to how ultrasound would physically perturb cells. Without an investigation into this upstream course of action, it would be difficult to formulate a coherent biophysical description of the cytomechanical events induced by low-intensity ultrasound.

In this article, we present new experimental insight into the cytomechanical effect of low-intensity ultrasound by providing novel direct observations on how cells would mechanically respond over the course of ultrasound exposure. Our investigation is founded upon the hypothesis that the mechanical bio-effects of low-intensity ultrasound are fundamentally initiated through physical perturbation of the cellular structure. To test this hypothesis, we have focused on unveiling how low-intensity ultrasound imposed on adherent cells would alter their morphology at both the membrane and nucleus levels. It should be emphasized that although the notion of low intensity has not been clearly defined in the medical ultrasound community, we shall work within the regulatory limits set forth by the U.S. Food and Drug Administration as normative thresholds: (i) 0.72 W/cm^2 for spatial-peak, time-averaged (SPTA) intensity, and (ii) 190 W/cm^2 for spatial-peak, pulse-averaged intensity (Duck 2007). As will be described later, all our experiments were performed below these acoustic exposure limits.

METHODS

Experimental apparatus for ultrasound-cell interaction analysis

Hardware description. This investigation was conducted using a composite apparatus that integrated two hardware units: (i) an ultrasound transmission setup whose waveform parameters can be arbitrarily defined; (ii) a confocal microscope that supported live *in situ* imaging of cellular response over the course of ultrasound exposure. Figure 1 is a block diagram of how these two hardware units, as well as the key components in each unit, are connected. As illustrated, the ultrasound transmission module comprised four serially connected components: a function generator (33120 A, Agilent Technologies, Santa Clara, CA, USA), a broadband amplifier (2100 L, Electronics & Innovation, Rochester, NY, USA), a 25.4-mm-diameter piston transducer with 1-MHz center frequency (Wuxi Beisheng Technology, Wuxi, Jiangsu, China) and a custom-made nosecone acrylic waveguide (75-mm height, 8-mm nose diameter, angled at 45°) that served as an acoustic collimator. During operation, the ultrasound transducer emitted pulses based on waveform parameters defined on the function generator. These pulses, after passing through the waveguide whose core was filled with water, impinged on cells seeded on the sample holder. The cellular response to ultrasound pulsing was then monitored *in situ* using the confocal microscope (LSM 710, Carl Zeiss, Jena, Germany). Note that the sample holder was filled with a buffer solution (composition to be described later) to facilitate acoustic coupling with the nose of the waveguide.

As illustrated in the lower-left inset of Figure 1, two types of sample holders were used in this work to accommodate different imaging configurations. The default type (type 1) was a 50-mm polystyrene dish with 1-mm base thickness (150288, Nunc, Roskilde, Denmark). This type of holder, used for imaging experiments performed on dry objective lens with millimeter-range working distance, has the advantage of exhibiting limited acoustic reflections at the point of incidence for ultrasound waves impinging at an angle (data discussed later). The second type of sample holder (type 2) was a customized design with a slab of 0.16-mm-thick cover glass (Vitromed, Basel, Switzerland) as the base layer. It was deployed in our 3-D high-contrast confocal scans done on oil immersion lens with sub-millimeter working distance (the type 1 holder cannot be used here because its base thickness was 1 mm). Note that the type 2 holder was essentially modified from the type 1 holder by first carving out a polystyrene base segment and resealing the resulting void with a slip of cover glass.

Alignment protocol. Before experiments began, calibration was performed to align the microscope's field of view with the ultrasound propagation path. In this procedure, a 4-mm pinhead was first affixed onto the center of the waveguide nose. The pinhead of the appended waveguide was then brought into physical contact with the surface of an empty polystyrene dish as illustrated in the upper right inset of Figure 1, and it was angled at 45° with respect to the surface normal to avoid blocking the microscope's optical path along the vertical axis. The