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● *Original Contribution*

QUANTIFICATION OF VERY LOW CONCENTRATIONS OF LEUKOCYTE SUSPENSIONS *IN VITRO* BY HIGH-FREQUENCY ULTRASOUND

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Abstract—Accurate measurement of very low cerebrospinal fluid (CSF) white blood cell (WBC) concentration is key to the diagnosis of bacterial meningitis, lethal if not promptly treated. Here we show that high frequency ultrasound (HFUS) can detect CSF WBC *in vitro* in concentrations relevant to meningitis diagnosis with a much finer precision than gold standard manual counting in a Fuchs-Rosenthal chamber. WBC concentrations in a mock CSF model, in the range 0–50 WBC/μL, have been tested and compared to gold standard ground truth. In this range, excellent agreement (Cohen's kappa [κ] = 0.78–90) (Cohen 1960) was observed between HFUS and the gold standard method. The presented experimental set-up allowed us to detect WBC concentrations as low as 2 cells/μL. HFUS shows promise as a low-cost, reliable and automated technology to measure very low CSF WBC concentrations for the diagnosis of early meningitis. (E-mail: javijimenezg@gmail.com) © **2016 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Cerebrospinal fluid, White blood cell, Cell count, High frequency ultrasound, Meningitis, Cytometry.**

INTRODUCTION

Accurate determination of the leukocyte (white blood cell [WBC]) concentration of cerebrospinal fluid (CSF) samples is clinically relevant in the diagnosis of meningitis (Kestenbaum et al. 2011; Manning et al. 2014). CSF is normally acellular, and the presence of low (5–25 WBC/μL) (Edwards et al. 2013; Greenberg et al. 2011) yet abnormal levels of WBCs raises clinical suspicion of meningeal infection. In the case of bacterial meningitis, early detection and immediate treatment are essential to improve prognosis and prevent lethal effects (Ishimine 2006). Currently, CSF samples are extracted through a lumbar puncture and WBC concentration can be obtained within an h. If diagnostic CSF WBC levels are exceeded and meningeal infection is suspected, treatment will be initiated. Because the blood–brain barrier becomes less permeable with age, diagnostic levels for CSF WBC concentration in meningitis are higher for patients younger than 29 d (>20–25 WBC/μL) than for patients between 30–90 d (>9 WBC/μL) and older than 90 d (>5 WBC/μL) (Seehusen et al. 2003; Edwards et al. 2013; Greenberg et al. 2011).

The clinical gold standard procedure to measure such low concentrations in CSF is the manual count of lumbar puncture samples with a Fuchs-Rosenthal cell counting chamber, which has associated measurement variability 30–50% at concentrations below 50 cells/μL, is operator dependent and time consuming (De Jonge et al. 2006; Kleine et al. 2012; Ngoyi et al. 2013; Zimmermann et al. 2011). Alternatively, automated cell counters are mostly designed for hematology analysis and hence, rely on a number of cell counts higher than those in CSF samples to provide a statistically confident measurement (De Jonge et al. 2006; Ongena et al.

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2010; Zimmermann et al. 2011). High frequency ultrasound (HFUS) has successfully been applied for cell characterization from the ultrasonic backscatter of individual cells (Baddour and Kolios 2006a). This technique has also been used to measure the integrated backscatter coefficient (BSC) from cells as an estimation of cell concentration in fluid samples. To compute BSC, the ultrasound signals centered at the acoustic focus are filtered with a Hamming window to suppress spectral lobes. Then, the Fourier transform is computed and squared to obtain the backscatter power spectrum S(f), which can be directly related to pressure intensities (Baddour et al. 2005). To calculate the absolute backscatter power spectrum, S(f) is normalized ($S_{NORM}[f]$) by dividing it by a reference spectrum ($S_{REF}[f]$) of the echo from a planar steel, brass or crystal reflector also centered at the focus to remove system and transducer transfer function (Baddour and Kolios 2006a; Elvira et al. 2016; Mercado et al. 2014). Nevertheless, to show relative differences between measurements obtained with the same equipment, normalization is not necessary and has not been performed herein. When the medium used in the experiment is different than that used to obtain the reference spectrum, the frequency-dependent attenuation coefficient of the medium used in the experiment is applied to $S_{NORM}(f)$ (Mercado et al. 2014; O'Donnell and Miller 1981). When a water-like medium is used, medium attenuation is negligible and may not be factored into the calculations (Kolios et al. 2004). While the frequency backscatter response allows modeling and characterization of the acoustic response of individual cells (Baddour et al. 2002), quantification of cell concentration is provided by the integrated $S_{NORM}(f)$ over the -6 dB bandwidth of the pulse. We refer to this integrated coefficient as the BSC power, although it is named differently in other studies (Baddour and Kolios 2006b; Mercado et al. 2014; O'Donnell and Miller 1981; Vlad et al. 2008). Other authors (Baddour and Kolios 2006b; Kolios et al. 2004; Tunis et al. 2005) used BSC to measure cell solution samples at concentrations above 50 cells/μL with a precision between tens to hundreds of cells/μL. A later work (Mercado et al. 2014) focusing on backscatter cell concentration measurement in engineered tissue showed a detection limit of 50 cells/μL. However, the authors aimed at providing a tissue characterization metric of the concentration of 10-μm cells in tissue, not fluids. To the best of our knowledge, the 0–50 cells/μL range has not been quantified with HFUS and is relevant in the diagnosis of early-stage meningitis.

In this paper, the capability of HFUS to measure WBC concentrations in the range 0–50 WBC/μL was explored. Series of samples of WBC concentrations in serum saline at 0.5 g/L plasma protein concentration were made to mimic healthy and early infected CSF. These samples were manually characterized at the routine analysis laboratory in University Hospital Clínico San Carlos in Madrid, Spain, using a Fuchs Rosenthal chamber. The coefficient of determination provided the degree of linear relationship between the methods. Agreement was measured with the Cohen's kappa coefficient for the different CSF WBC concentrations for meningitis diagnosis. A Bland-Altman analysis determined the bias and 95% confidence interval (CI) of the paired measurements differences between methods.

MATERIAL AND METHODS

Preparation of cell solutions

Blood samples from 20 patients with leukocytosis arriving at the routine analysis laboratory in University Hospital Clínico San Carlos were collected in EDTA-K3 tubes (BD Vacutainer, BD, Franklin Lakes, New Jersey, USA). Approval for the study was obtained from the hospital's Ethics Committee and all volunteers gave their informed consent. The tubes were centrifuged at 400g for 15 min and between 1–2 mL of plasma from each EDTA tube were used to create a pool of plasma. A method based on the modification of Weichselbaum's (1946) was used to measure protein pool in the pool with an AU5800 analyzer (Beckman-Coulter, Pasadena, California, USA). Saline serum (B. Braun Medical, Melsungen, Germany) was added to obtain a final solution volume of 50 mL at 0.5 g/L protein level. This protein matrix constituted the healthy model of CSF. From each EDTA tube, 1 mL of buffy coat was pipetted out with a Pasteur pipette (Copan, Brescia, Italy) and transferred to a Wintrobe tube (Fisher Scientific, Madrid, Spain). Ten Wintrobe tubes were then centrifuged at 400 g for 15 min and buffy coat components were separated in four distinct layers: plasma only, platelets enriched plasma, a leuko-platelet layer and an erythrocytes layer. Plasma was discarded and the platelet-enriched plasma was saved to assess the impact of platelets in the ultrasound measurement. For each tube, the leuko-platelet layer was pipetted out and diluted in 1 mL of the protein matrix. An additional centrifugation at low speed (40 g for 5 min) was performed to separate the majority of platelets. Supernatant was eliminated and the pellet was re-suspended with 1 mL of the protein matrix. From this stock suspension, cell dilutions were prepared by diluting high-concentration samples that had been previously measured by a hematology system (Advia 120, Siemens Healthcare, Erlangen, Germany) to obtain concentrations in the range 0–50 WBCs/μL. The measurement error of these systems for concentrations above 100 cells/μL is 5%–10% (De Jonge et al. 2006; Ongena et al. 2010; Zimmermann et al. 2011).