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● *Original Contribution*

PROTOCOL FOR ROBUST *IN VIVO* MEASUREMENTS OF ERYTHROCYTE AGGREGATION USING ULTRASOUND SPECTROSCOPY

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Abstract—Erythrocyte aggregation is a non-specific marker of acute and chronic inflammation. Although it is usual to evaluate this phenomenon from blood samples analyzed in laboratory instruments, *in vivo* real-time assessment of aggregation is possible with spectral ultrasound techniques. However, variable blood flow can affect the interpretation of acoustic measures. Therefore, flow standardization is required. Two techniques of flow standardization were evaluated with porcine and equine blood samples in Couette flow. These techniques consisted in either stopping the flow or reducing it. Then, the sensibility and repeatability of the retained method were evaluated in 11 human volunteers. We observed that stopping the flow compromised interpretation and repeatability. Conversely, maintaining a low flow provided repeatable measures and could distinguish between normal and high extents of erythrocyte aggregation. Agreement was observed between *in vivo* and *ex vivo* measures of the phenomenon (R^2 = 82.7%, p value < 0.0001). These results support the feasibility of assessing *in vivo* erythrocyte aggregation in humans by quantitative ultrasound means.

Key Words: **Quantitative ultrasound, backscatter coefficient, spectral analysis, erythrocyte aggregation, flow phantom study, *in vivo* measures, reliability.**

INTRODUCTION

Erythrocyte aggregation refers to the reversible tendency of mammalian red blood cells (RBCs) to associate under low blood flow or stagnation conditions (Baskurt et al. 2011). RBC hyper-aggregation has been extensively reported in various pathophysiological conditions, such as cancers, diabetes mellitus and cardiovascular diseases. In particular, hyper-aggregation is often associated with inflammatory disorders, as some proteins released in the bloodstream, notably fibrinogen, enhance inter-RBC attraction forces (Weng et al. 1996a). Consequently, indicators of erythrocyte aggregation are commonly used in clinical research as a surrogate marker of inflammation (Berliner et al. 2005; Reggiori et al. 2009).

Several techniques have been proposed for the quantitative measurement of erythrocyte aggregation. In general, the measure relies on laboratory-based instruments requiring blood sampling and the use of anticoagulant. Indices based on erythrocyte sedimentation rate (Fåhraeus 1929), blood viscosity (Chien et al. 1967) or light transmission and scattering (Baskurt et al. 1998) are used as indicators of the degree of RBC aggregation. Particularly, the laser aggregometer is still the most accepted instrument for RBC aggregation measurements (Baskurt et al. 2009; Zhao et al. 1999). The key advantage of this instrument is that the measure is done under well-controlled conditions, thus reducing the effect of confounding factors (*i.e.,* shear rate, hematocrit, sedimentation and temperature).

In vivo assessment of RBC aggregation has also been proposed, mostly using ultrasound approaches (Rouffiac

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et al. 2004; Tripette et al. 2013, 2015). The main aim of *in vivo* measurements is to continuously monitor the acute inflammation response in patients under critical care evaluation, notably those at risk of septicemia (Fernandes 2013; Tripette et al. 2013). The most used ultrasound technique is based on the measurement of a fundamental acoustic property of blood: the backscatter coefficient (BSC). The BSC is a quantitative value that reflects the capacity of a tissue to return acoustic energy at a certain frequency (Shung 2005). At any physiologic hematocrit, the BSC of blood increases in amplitude as a result of erythrocyte aggregation (Foster et al. 1994; Yu and Cloutier 2007; Yuan and Shung 1988). The main advantage of the BSC is that it is independent of the ultrasound system characteristics, so reported values can be compared between laboratories (Anderson et al. 2010). It is a common practice to summarize the frequency-dependent BSC with descriptive parameters, which is usually represented by a spectral curve fitted to scattering models (Franceschini et al. 2008; Yu and Cloutier 2007) or by taking the slope of the curve (Scheipers 2009). Other ultrasound techniques, such as power Doppler (Cloutier and Shung 1992) and envelope statistics (Destrempes et al. 2016), have also been proposed to quantify RBC aggregation.

Despite several efforts, there are still a few challenges for *in vivo* measurements by ultrasound. In contrast to laboratory-based *ex vivo* techniques, *in vivo* measurements imply a lack of control over factors such as the blood temperature, hematocrit and flow shear rate. Most authors implicitly consider that changes in body temperature are minimal and neglect this effect, which is acceptable under changes up to a few degrees Celsius (Baskurt et al. 2011; Neumann et al. 1987). On the other hand, hematocrit changes can cause significant differences in measurements and are usually corrected for or considered constant unless a hemorrhagic condition prevails.

The main confounding factor for *in vivo* assessment of RBC aggregation is the flow shear rate. Shearing forces are created by flowing blood traveling at different velocities depending on the position in the vessel. RBC aggregation typically occurs at shear rates between 0.0001 and 100 s^{-1}, with more aggregation present at the lowest range (Chien et al. 1967). In conditions such as diabetes mellitus, shear rates above 100 s^{-1} may be necessary to disrupt pathologic RBC aggregates (Cloutier et al. 2008). At high shears near the vessel wall, aggregates partially or totally dissociate (Fig. 1). Conversely, low shear rates can be found close to the longitudinal axis of a vessel or in recirculation zones, which enhance RBC aggregation.

A minimum shear rate is necessary to promote aggregation as collision efficiency between RBCs is reduced

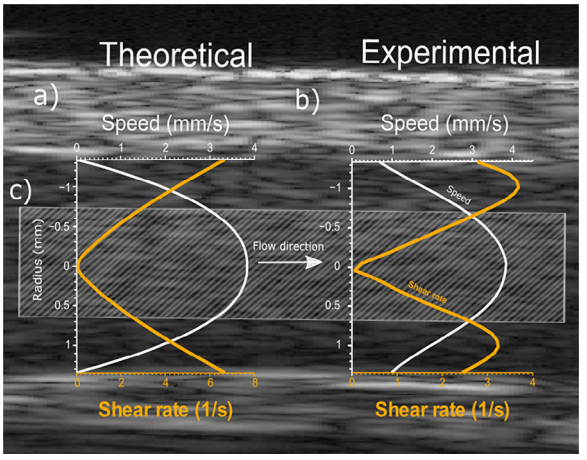

Fig. 1. Examples of *in vivo* velocity and shear rate profiles in vein flow. (a) Theory predicts a blunted parabolic velocity profile created by the non-Newtonian behavior of blood. Shear rate is zero at the central axis of the vein and maximum at the wall. (b) Experimental flow profile calculated with the particle image velocimetry method (see details below). Artifacts close to the wall are caused by the lack of precision of the estimation under 1 mm/s because of the use of a spatial filter. (c) Region of interest for spectral analysis (see details below). This area contains a median shear rate of about 3 s^{-1}.

under flow stagnation (Shehada et al. 1993). Flow pulsatility in arteries can also modulate the level of aggregation (Cloutier and Shung 1992; De Kroon et al. 1991). With *ex vivo* Couette systems made of concentric cylinders, as typically used in rheology studies, steady or pulsatile flow can be obtained and shearing forces can be precisely controlled. Couette devices allow the time course of RBC aggregation to be followed from a disaggregated state at a high shear rate (*i.e.*, at a high relative rotation speed of concentric cylinders), or permit evaluation of the impact of a specific shear rate on aggregate size (Nguyen et al. 2008).

Two approaches have been proposed for shear rate adjustment in *in vivo* RBC aggregation measures with ultrasound: either reducing the blood flow by clamping the vessel or no control at all (*i.e.*, natural flow). In porcine experiments (Rouffiac et al. 2004; Tripette et al. 2013), an adjustable clamp was applied downstream of the ultrasound transducer to reduce the blood flow. Low-velocity profiles could give a relatively narrow dispersion of shear rates. In those studies, ultrasound acquisitions were done during the first minute after stabilization of the flow. In human (Tripette et al. 2015) and rabbit (Yu et al. 2011) studies, the venous flow was not controlled, but the maximum velocity was measured to estimate the mean shear rate assuming Poiseuille flow.

The objective of this study was to describe an ultrasound protocol to assess the erythrocyte aggregation level *in vivo* that can be used in a clinical context.