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Acute hydrodynamic damage induced by SPLITT fractionation and centrifugation in red blood cells

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ABSTRACT

Though blood bank processing traditionally employs centrifugation, new separation techniques may be appealing for large scale processes. Split-flow fractionation (SPLITT) is a family of techniques that separates in absence of labelling and uses very low flow rates and force fields, and is therefore expected to minimize cell damage. However, the hydrodynamic stress and possible consequent damaging effects of SPLITT fractionation have not been yet examined. The aim of this study was to investigate the hydrodynamic damage of SPLITT fractionation to human red blood cells, and to compare these effects with those induced by centrifugation. Peripheral whole blood samples were collected from healthy volunteers. Samples were diluted in a buffered saline solution, and were exposed to SPLITT fractionation (flow rates 1–10 ml/min) or centrifugation (100–1500 *g*) for 10 min. Cell viability, shape, diameter, mean corpuscular hemoglobin, and membrane potential were measured. Under the operating conditions employed, both SPLITT and centrifugation maintained cell viability above 98%, but resulted in significant sublethal damage, including echinocyte formation, decreased cell diameter, decreased mean corpuscular hemoglobin, and membrane hyperpolarization which was inhibited by EGTA. Wall shear stress and maximum energy dissipation rate showed significant correlation with lethal and sublethal damage. Our data do not support the assumption that SPLITT fractionation induces very low shear stress and is innocuous to cell function. Some changes in SPLITT channel design are suggested to minimize cell damage. Measurement of membrane potential and cell diameter could provide a new, reliable and convenient basis for evaluation of hydrodynamic effects on different cell models, allowing identification of optimal operating conditions on different scales.

1. Introduction

Obtaining red blood cell (RBC) fractions with high purity, yield, and viability is important for blood banks and transfusion medicine. Blood bank processing, which employs centrifugation, imposes mechanical forces on erythrocytes, which, as with any manufacturing process, may contribute to hemolysis and sublethal damage of the remaining cells, limiting their normal function and compromising their therapeutic effectiveness [1]. Split-flow fractionation (SPLITT) is a family of techniques that separates differently sized particles in thin, rectangular channels using gravitational and flow fields aligned perpendicularly to each other [2]. SPLITT fractionation allows continuous processing of whole blood without antibody labelling to obtain fractions of RBC, platelets and plasma proteins in short time with high purity and viability [3]. The absence of labelling and the use of very low flow rates and force fields are expected to induce very low shear stress and minimize cell damage, making it an attractive technique for large-scale separations [4].

RBCs, which in vivo are suspended cells that experience mechanical stress, have been recommended as a standard cell model for comparative assessment of the potential for damage in various hydrodynamic environments [5,6]. Since erythrocytes are

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enucleated post-mitotic cells and unable to multiply, the injurious effects of mechanical stress cannot be masked by the adaptive changes of cell growth and multiplication, as is observed in other cell types [7]. Additionally, red blood cell shape is extremely sensitive to metabolic and medium changes, and to mechanical stress [6]. Although it has been documented that different biomedical devices and bioreactors generate morphological changes in RBCs that are attributable to mechanical stress [8,5,9–11], the hydrodynamic stress and consequent damaging effects of SPLITT fractionation have not been yet determined, partly because it is assumed that the low flow rates and force fields used in this technique do not generate significant mechanical stress [12,4]. To test the apparent innocuousness of SPLITT separation, it is necessary to use very sensitive indicators for assessing cell damage. The aim of this study was to investigate the hydrodynamic damage of SPLITT fractionation on human red blood cells, and to compare these effects with those induced by centrifugation.

2. Materials and methods

2.1. Sample collection and preparation

This study was approved by the institutional Ethics Committee of the Universidad Nacional de Colombia and followed current protocols for research on humans and handling of biological samples (Declaration of Helsinki). Blood samples were collected in EDTA from healthy volunteers. Since the interactions between cells can generate changes in the flow profile in the SPLITT channel, it was necessary to prepare highly diluted cell suspensions (hematocrit 0.5%) using a saline solution containing (in mM): 140 NaCl; 5 KCl; 1$CaCl_2$; 1 $MgCl_2$; 10HEPES; 10 glucose; pH 7.4; 292 ± 1 mOsm/l; viscosity 0.00131 Pa·s; density 1007.1 kg/m^3 (chemicals were obtained from Sigma-Aldrich, St. Louis, MO, USA). This solution was used both for diluting samples and as a carrier fluid. The osmolarity of the solution was measured with a vapor pressure osmometer (Wescor Model 5600, ELITechGroup, Paris, France), the dynamic viscosity was measured with a cone and plate rheometer (Bohlin C-VOR 200, Malvern Instruments, Malvern, UK), and density was measured with a pycnometer. Since the use of plasma has been shown to be protective against mechanical damage in red blood cells [5], experiments with autologous plasma supplementation (final concentration of 10% v/v) were also conducted (311 ± 2 mOsm/l; 0.0016 Pa·s). Assays were performed at room temperature ($20 \pm 2\,^{\circ}C$).

2.2. SPLITT channel

A 186 mm long (L), 20 mm wide (B) and 0.48 mm deep (D) SPLITT channel was used. The channel was fabricated at the PMMH laboratory by alternating plates of Plexiglas (polymethyl methacrylate) and spacers of Mylar (polyethylene terephthalate) (Fig. 1A). The sample was injected through the inlet labelled *a* with a constant flow rate $Qa = 0.2$ ml/min, while the inlet *b* was fed with the carrier fluid at a variable flow rate Qb, so the total flow rate $Q = Qa + Qb = Qaí + Qbí$. In order to use the device in a hydrodynamic test to evaluate its maximal potential for damage, output fractions were collected only from the lower outlet labelled *b*, by keeping the outlet *a* closed. Total flow rates employed ranged from 1.5 to 10.0 ml/min, and were controlled with syringe pumps (Harvard Apparatus, Holliston, MA). Outlet fractions and non-treated controls were processed immediately for the measurement of morphological indicators and membrane potential.

2.3. Centrifugation

A bench centrifuge with a 45° fixed-angled rotor (Sorvall Biofuge Primo R, Thermo Scientific, Waltham, MA) was used to centrifuge 10 ml of cell suspension in polypropylene centrifuge tubes (Falcon™, BD Biosciences, San Jose, CA, USA) at $20 \pm 2\,^{\circ}C$ for 10 min at 100, 200, 400, 900 or 1500 g with no brake, equivalent to total centrifugal g-second values of 6, 12, 240, 540, or 900×10^4 g·s. respectively These centrifugation parameters are similar to those used in mononuclear blood cell isolation with Ficoll®-Paque and in blood bank processing [13].

2.4. Hemoglobin release

After one pass through the SPLITT channel, output fractions were collected and centrifuged at 2750g for 15 min at room temperature to obtain supernatants. In contrast, after centrifugation assays, supernatants were collected directly with no additional centrifugation. Supernatants thus obtained were frozen and stored at $-30\,^{\circ}C$ for subsequent analysis. Hemoglobin released in the supernatant was measured via optical absorbance at 414 nm using a microplate reader (Ultramark 550, Bio-Rad Laboratories, Hercules, CA, USA) with the Harboe method [14,15]. A calibration curve was constructed using a standard of 100% hemolysis by hypotonic lysis with distilled water and 12 1:2 serial dilutions until 0.024% hemolysis.

2.5. Cell morphology

Non-treated negative controls and output fractions were transferred into 24 well culture plates to obtain micrographs with an inverted light microscope (40× objective) equipped with an image acquisition system (AxioCam, Carl Zeiss MicroImaging, Göttingen, Germany). The captured images were stored in *.zvi* format and subsequently analyzed with the software AxioVision (SE64, Rel. 4.8; Carl Zeiss MicroImaging, Göttingen, Germany) to determine cell shape. Using the 40× dry objective, a portion of the image field was selected with no overlapping red blood cells, and 10 fields were counted using the zigzag method until completing 100 cells per experiment. The amount of echinocyte-discocyte transformation was determined by calculating the morphological index I Eq. (1), an indicator of extent of morphological change, by incorporating the classification of echinocytes from type I to III according to the number of spikes per cell [16]:

$$I = \lceil 1 \times \text{echinocytesI} \rceil + \lceil 2 \times \text{echinocytesII} \rceil + \lceil 3 \times \text{echinocytesIII} \rceil \quad (1)$$

Since the morphological index I increases with incubation time [17], a period of 2–3 h was standardized when performing the experiment.

2.6. Cell diameter

The cell diameter of one hundred cells per experiment (from 14 independent assays) was measured from photomicrographs by image analysis. At the beginning of each experiment, etched lines on a calibrated stage micrometer were used to set the μm/pixel conversion factor. Using the AxioVision image analysis software, a line was traced connecting the outer dark edges at the rims of each cell; only the horizontal diameter was measured for cells on edge, whereas for the cells lying flat the maximum diameter was recorded.

2.7. Mean corpuscular hemoglobin concentration

Total hemoglobin concentration (Hb; g/dl) was measured by the azide methemoglobin method, and hematocrit (Hct), by impedance cytometry using an automated haematological analyzer (Sysmex®