Hemorheological measurements in experimental animals: further consideration of cell size – pore size relations in filtrometry

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Abstract

Micropore filtration of dilute red blood cell (RBC) suspensions is a widely known method for determining red blood cell deformability. Use of this method for cells from various laboratory animal species does require considering the effects of the cell size to pore size ratio and of suspension hematocrit. In general, previous animal studies have utilized 5% hematocrit suspensions and five micron pores, and thus conditions similar to human clinical laboratory practice. However, when used for repeated sampling from small laboratory animals or for parallel multiple samples from different sites in large laboratory animals, the volume of blood sampled and hence the hematocrit of the test suspension may be limited. Our results indicate that hematocrit levels yielding stable values of RBC pore transit time are pore size and species specific: three micron pores = 2–5% for dog and 3–5% for rat; five micron pores 3–5% for dog and 1–5% for rat. An analytical approach using a common expression for calculating transit time is useful for determining the sensitivity of this time to hematocrit alterations and hence to indicate hematocrit levels that may be problematic.

Keywords: red blood cell deformability, micropore filtration, filtrometry, filtration rate, transit time, cell to pore size ratio

1. Introduction

Measurement of red blood cell deformability can be determined by several methods, including bulk filtrometry, pore transit analysis, laser-diffraction ellipsometry or ektacytometry, micropipette aspiration, and single cell techniques of micro-flow devices (Hardeman et al., 2007; Lee et al., 2007; Baskurt et al., 2009).

Bulk filtrometry is an old but still a widely used method to describe red blood cell deformability via filtration of red cell suspensions through a micropore filter, often at a constant pressure gradient (Dormandy et al., 1985). The method is relatively cheap, but it has several limitations because of the bulk filtration itself (blocked pores by white blood cells, measurement of relative parameters, dependence on pore size and hematocrit, etc) (Reinhart et al., 1984; Matrai et al., 1985; Nemeth et al., 2006a). However, several clinical and experimental research studies - over decades till date - shows its usefulness for detecting indicative changes in red blood cell deformability in various pathophysiological processes (e.g., Nash, 1990; Bernat et al., 2005; Koltai et al., 2006; Nemeth et al., 2006b; Peto et al., 2007) and to approximate the microcirculatory effects (Lipowsky et al., 1993; Losco et al., 2001). Using this method in animal experiments raises two challenges to carrying out the tests: 1) the available versus the required volume of blood samples, mainly in small laboratory animals; 2) the cell size to pore size or cell volume to pore volume ratio.

The usual hematocrit of the red blood cell suspension is 5% in clinical and in most published research studies. However, to obtain a sufficient amount of a 5% hematocrit red blood cell suspension requires a volume of whole blood that can not be tolerated for small laboratory animals (e.g., mice, rats). Therefore, a reduction of suspension hematocrit is sometimes suggested, even though filtration properties may differ with the dilution (Schmalzer et al., 1983; Reinhart et al., 1984).

The pore size to cell size ratio and/or the pore volume to cell volume is also an important parameter since filtration values differ if the pore size is constant but the cell size is changed (i.e., blood cells from various animal species). Similar uncertainties also occur when testing the same sample with various pore sized filters. Thus, the sensitivity of the filtration tests is strongly depends on the cell size to pore size ratio (Reinhart et al., 1984; Baskurt et al., 1996).
In previous studies with small laboratory animals, experiments utilized 1% hematocrit red blood cell suspensions in order to permit repeated studies in the same animal (Nemeth et al., 2006b; Miko et al., 2006). However, in a pilot study, red blood cell suspensions at 1–5% hematocrit were prepared from the same sample and filtration parameters were determined using a bulk filterometer (Carat FT-1 filterometer) based on St. George’s technique (Dormandy et al., 1985). We found that the ratio of suspension filtration rate to buffer rate slightly decreased, and consequently relative cell transit time moderately increased, when moving from 1 to 5% hematocrit suspensions. Nevertheless, it was concluded that although filtration parameters values at 1% are not equal to those at 5% hematocrit, it is possible to use 1% suspension with good reproducibility (Nemeth et al., 2004).

Later studies of mongrel dog, rat and mouse blood cell suspensions at 1–5% hematocrit were carried out using the commercially available three and five micron pore sized polycarbonate filters in a Carat FT-1 filterometer device (Nemeth et al., 2006a). As anticipated, the results indicated that the initial relative filtration rate decreased from 1 to 5% with different manner. However, the calculated value of relative cell transit time was affected differently by hematocrit in the three and five micron filters and in various animal species: 1) using five micron filters for mice or rats red cells yielded the same changes of relative cell transit time (i.e., decreased with decreasing hematocrit), whereas with three micron filters this time slightly decreased between 5 and 3% then increased with the highest values at 1% hematocrit suspension; 2) in mongrel dogs similar rise in RCTT could be observed even when using five micron filters with 1 and 2% hematocrit suspensions (Nemeth et al., 2006a).

It is supposed that the above phenomena are being affected by geometric factors (i.e., cell size to pore size ratio), and hence by cellular properties and their differences among various animal species.

The present study was designed to extend these observations to red cells from inbred beagle dogs in order to determine whether similar effects of pore size and suspension hematocrit occur. In addition, the resulting data were used for more detailed analyses of the filtration parameters, since the optimal conditions for bulk filterometry on laboratory animals blood samples have not been completely clarified yet that would be useful as a recommendation for future studies.

2. Materials and methods

2.1. Animals and preparation of samples

The experiments were approved by the Committee of Animal Research at University of Debrecen (permission number 13/2003 UDCAR and 37/2007 UDCAR). In morning hours blood samples were withdrawn from five beagle dogs (body-weight: 13.25±1.76 kg) via puncture of the cephalic vein using a closed sampling system (sodium-heparin, 143 IU, BD Vacutainer® 7 ml, Belliver Industrial Estate, UK).

Blood samples were centrifuged at 2,500 g for 10 minutes, the plasma and ‘buffy coat’ removed, and the cell suspensions washed twice in normal phosphate buffered saline (PBS, pH=7.4, 285 mOsm/kg). Hematological parameters for the suspensions were determined by a Sysmex F-800 microcell counter (TOA Medical Electronics Co., Japan), following which each sample was divided into five parts and PBS was added to adjust the hematocrit of the suspensions to 1, 2, 3, 4 or 5%.

2.2. Laboratory investigations

2.2.1. Determination of hematological composition and viscosity of cell suspensions

A Sysmex F-800 microcell counter (TOA Medical Electronics Co., Ltd., Japan) was used to determine the final hematological composition of each dilute red cell suspension. Suspension viscosities were determined at 37°C using a Haevisit-40 capillary viscometer (Hemorex Ltd., Hungary).

2.2.2. Filtration tests of red blood cell suspensions

A Carat FT-1 filterometer (Carat Ltd., Hungary) was used for determination of red blood cell suspension filtration parameters (Dormandy et al., 1985). Measurements were carried out within two hours after blood withdrawal and were performed at room temperature (22±1°C) (ICSH Expert Panel on Blood Rheology, 1986; Bernat et al., 2005; Baskurt et al., 2009).

The red blood cell suspensions were filled into the filtermeter chamber, which space is divided into two parts by the polycarbonate filter. Two types of polycarbonate filter membranes were used for each sample: pore diameter of three or five microns (Nuclepore membranes, Whatman Inc., filter diameter=13 mm, average pore length=10 microns). When the suspension starts to flow by opening a little tap resulting in a constant filtration pressure of four cm of water, the position of the fluid is determined by four pairs of light sources and photodetectors. The filtermeter is interfaced to a computer which automatically determines sequential flow rates via four pairs of light sources and photodetectors, then extrapolates to zero volume filtered in order to determine the initial relative filtration rate (IRFR). Using the IRFR and suspension hematocrit (Hct) the relative cell transit time (RCTT) is calculated as follows:

\[
\text{RCTT} = \left(\frac{1}{\text{IRFR}} - 1\right) / \text{Hct} + 1
\]

Note that if the suspension flows more slowly through the filter (decreased red blood cell deformability), IRFR decreases and the calculated RCTT increases (i.e., greater time for cells to traverse the filter pores).

2.3. Statistical analyses

Statistical analyses were carried out with ANOVA test
Table 1. Viscosity and hematological parameters for diluted red cell suspensions

<table>
<thead>
<tr>
<th>Measured Parameter</th>
<th>Suspension Hematocrit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1%</td>
</tr>
<tr>
<td>Viscosity [mPa.s]</td>
<td>0.68±0.01</td>
</tr>
<tr>
<td>Red blood cell count [x10^6/μl]</td>
<td>0.17±0.02</td>
</tr>
<tr>
<td>Hematocrit [%]</td>
<td>1.03±0.05</td>
</tr>
<tr>
<td>Mean corpuscular volume [fl]</td>
<td>71.3±1.75</td>
</tr>
<tr>
<td>Leukocyte count [x10^3/μl]</td>
<td>0.13±0.06</td>
</tr>
<tr>
<td>Platelet count [x10^3/μl]</td>
<td>41±12.5</td>
</tr>
</tbody>
</table>

means S.D.

(Bonferroni’s method) and Student t-tests. Linear regression analysis was used to determine relations between parameters. A p value of <0.05 was considered significant.

3. Results

3.1. Composition of blood cell suspensions

Table 1 shows viscosity values and hematological parameters for the beagle dog red cell suspensions. The hematocrit values were consistent with the nominal values (i.e., 1, 2, 3, 4, and 5%), with suspension viscosity and red blood cell count increasing with hematocrit. Red cell mean corpuscular volume (MCV) was not affected by preparing the suspensions and did not change markedly from the control value of 71.9±1.7 fl. White blood cell and platelet counts were markedly reduced compared to control blood samples: residual leukocyte value <1% of 11.96±0.5×10^6/μl, residual platelet count <10% of 464±136×10^3/μl.

3.2. Alterations of initial relative filtration rate (IRFR) and relative cell transit time (RCTT)

Fig. 1 presents relative filtration rate (IRFR) and relative cell transit time (RCTT) values, obtained using both three and five micron pore filters, for red cell suspensions having various levels of hematocrit. Inspection of these results indicates: 1) Using five micron pore filters, IRFR decreased and RCTT increased with increasing hematocrit, and both the IRFR and RCTT for 1% suspensions differed significantly when compared to the “standard” 5% suspensions (p<0.009, p<0.001, respectively); 2) With three micron pore filters, IRFR also decreased with increasing hematocrit, whereas RCTT exhibited a biphasic response such that it was higher for the 1 and 2% hematocrit suspensions than the 5% hematocrit suspensions. Three micron pore IRFR values of the 1, 2 and 3% suspensions were significantly greater compared to 5% (p<0.001, p<0.022, p=0.045, respectively). Linear regression results for IRFR and RCTT were significant only for the five micron pore data (p<0.05, p<0.01 respectively).

Fig. 2 summarizes the relative changes of both IRFR and RCTT compared to the standard 5% suspension and hence a value of unity indicates no change from 5% values. Note that for three micron filters the relative changes of IRFR were >1 and larger than for five micron filters, and that five micron RCTT relative changes were less than unity below 3% hematocrit.

3.3. Analysis of IRFR-RCTT relations for beagle dog and rat red cell suspensions

Given the mathematical relationship between IRFR and RCTT (see Equation 1 above), it is possible to construct a family of curves with suspension hematocrit as the variable.
Fig. 2. Relative values of IRFR and RCTT for beagle dog red blood cell compared to the 5% suspension using 3 and 5 μm pore size filters at various suspension hematocrits (data are from Fig. 1).

Fig. 3. IRFR and RCTT mean values at different hematocrits for beagle dogs and outbred rats (Nemeth et al., 2006a) measured using three or five μm pore size filters. Solid lines represent predicted IRFR-RCTT relations at various hematocrits (1–5%) calculated using the relation: RCTT = [(IRFR)^(-1) - 1]/Hct + 1. The small insert presents an enlarged portion of IRFR-RCTT curves and results for five μm pore filters. As orientation, the normal values of MCV of CD = 53.68 ± 2.19 f; MCV of beagle = 70.03 ± 3.26 f, measured by a Sysmex F-800 microcell counter (Nemeth et al., 2009a).

Fig. 3 presents such a set of curves for suspensions having hematocrits of 1 to 5%. The IRFR-RCTT relations are seen to be non-linear, with the degree of curvature and sensitivity of RCTT to IRFR greatest for the 1% suspension. Thus for lower values of suspension hematocrit and IRFR, a relatively small change of the experimentally measured IRFR results in a substantially larger change of the calculated value of RCTT.

Fig. 3 also shows the mean IRFR and RCTT values for the present study and for previous rat data (Nemeth et al., 2006a) superimposed on the family of curves; Fig. 3B has IRFR values appropriate for the five micron results. Dealing first with the three micron results, it can be seen that rat data lie on the predicted hematocrit curves over the entire 1–5% range, while dog data are only in agreement for 3, 4 and 5%; dog data lie below the predicted curves at both 1 and 2% hematocrit. It is notable that when using three micron filters, fairly stable values of RCTT for dogs are obtained over the range of 2 to 5% hematocrit, whereas rat red cell values show similar stability only for 3, 4 and 5%; three micron pore size RCTT values for rats are elevated for the 1 and 2% suspensions as are the dog data at 1%.

Using five micron pore size filters results in higher IRFR values for both species compared to those obtained with three micron pores (Fig. 3). Again, as seen for three micron pores: 1) rat data lie on the predicted hematocrit curves over the entire 1–5% range; 2) dog data only agree over 3–5%, with the 1 and 2% results markedly below the predicted curves. Comparing the values measured at 5% hematocrit for three and five micron filters, one can conclude that RCTT values are essentially identical for rats and dogs, whereas at lower hematocrits (e.g., 1 or 2%) RCTT values for rats are higher than for beagle dogs.

4. Discussion

Although the development of new or improved measuring techniques has greatly aided hemorheological research and has provided insight regarding the importance of rheologic abnormalities in many clinical states, there remain several unresolved issues relating to species-dependent hemorheological characteristics (e.g., Usami et al., 1969; Chien et al., 1971; Chen and Kaul, 1994; Baskurt, 1996; Plasenzotti et al., 2004; Nemeth et al., 2009a and 2009b). Since small animals are currently being used for a variety of research studies, it is critical that comparative data be available in order to determine the usefulness of rheologic methods as well as the limits and potential sources of error associated with these methods.

Devices based on the filtration behavior of red cell suspensions are widely used for assessing blood cell deformability in different clinical states as well as in experimental research. In general, these devices measure the flow rate of dilute red cell suspensions through three or five micron diameter pores in special microprobe filters, then either report this rate or calculate the time required for a cell to traverse the pore (i.e., the transit time). It is notable, however, that the term “deformability” has no units and merely means the ability of a red cell to adopt a new shape in response to deforming forces. Thus, results using filtration methods to assay red cell deformability and the sensitivity of methods to rheologic changes may differ depending on the experimental conditions (Reinhart et al., 1984; Dombandy et al., 1985; Mátrai et al., 1985; Koutsouris et al., 1989).

Employing a fritrometer based on the St.George’s technique (Dombandy et al., 1985), we have previously mea-
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sured the filtration behavior of different hematocrit suspensions for various animal species using the commercially available three and five micron pore size filters (Nemeth et al., 2006a). These investigations were focused on the question of whether it is possible to find an 'optimal' set of conditions for various laboratory animal species, with particular attention given to the minimum volume of blood required and the cell size to pore size ratio. The prior findings indicated that RCTT values for three micron pore filters tended to decrease with increasing hematocrit for mongrel dog, rat and mouse erythrocytes; with five micron pores, RCTT values for rat and mice cells also tended to increase while a decrease was observed for dog cells (Nemeth et al., 2006a). Our current RCTT results for RBC from beagle dogs (Fig. 1) differ qualitatively from these earlier mongrel dog data: prior RCTT values decreased with increasing hematocrit for both three and five micron pores (Nemeth et al., 2006a), whereas herein a biphasic effect was seen using three micron pores and a slight increase was noted for five micron pores. The reasons for these findings are not yet clear, especially since suspension viscosity and hematocrit, MCV, and leukocyte and platelet counts did not differ between the mongrel dog and beagle dog studies.

An interesting analytical aspect of the present study is the calculation of a family of IRFR-RCTT curves for various hematocrits, then examining experimental results vis-à-vis these curves. These curves were plotted by the mathematical relationship between IRFR and RCTT using the suspension hematocrit as the variable (see Equation 1 above). The IRFR-RCTT curves help to explain the background of the distortion effect of usage of low hematocrit suspension. As shown in Fig. 3 for three micron pores, beagle dog RCTT results were on the appropriate curve for 3, 4 and 5% hematocrit but were below the lines for 1 and 2%; rat erythrocyte data were in agreement with the correct curve over the entire hematocrit range. Similar behavior is seen for five micron pores (i.e., agreement for beagle dog only for 3–5%, complete agreement for rat), with the dog cells having even a larger divergence from the curves at 1 and 2%.

It is important to recognize that data being congruent with the appropriate curve is a necessary but not sufficient condition for stable values of RCTT: for three micron pores, rat red blood cells at 1 and 2% hematocrit are clearly elevated above the mean for these cells at 3–5%, whereas with five micron pores rat RCTT values are stable and congruent with the proper curve over the 1–5% hematocrit range. RCTT data for beagle dogs at 1 and 2% hematocrit are also elevated with three micron pores yet are below the mean with five micron filters (Fig. 1 and 2). Since RCTT is a measure of red blood cell deformability (i.e., the ability of the entire cell to adopt a different shape in response to deforming forces), its value would be expected to be constant for a given population of cells. Potential explanations for non-stable RCTT data include: 1) Incorrect suspension hematocrit, yet both the prior (Nemeth et al., 2006a) and current study employed hematocrit that agreed very closely with the nominal values (Table 1); 2) Problematic IRFR values due to temporary or permanent pore clogging by non-filterable cells, white blood cells and clumped platelets (Chien et al., 1983; Matrai et al., 1985; Lisovskaya et al., 1998), although both white cell and platelet counts were reduced and the use of an IRFR procedure minimizes the effects of pore plugging as filtration proceeds (Dormandy et al., 1985); 3) Hemodynamic effects of cell-cell and cell-pore interactions (Schnalzer et al., 1983; Reinhart et al., 1984; Lindmark and Engstrom, 1996), yet these effects would be expected be greater at higher rather than at lower hematocrits; 4) Alterations of red blood cell morphology (i.e., echinocye formation), since transformation of the normal biconcave shape to a crenated disc or sphere occurs more easily at low hematocrits (Nemeth et al., 2006a) and since echinocytic red blood cells have reduced deformability (Meiselman, 1978 and 1981; Pfafferott et al., 1982).

Regardless of the specific mechanisms responsible for the variations seen at 1 and 2% hematocrit, it is clear that the use of filtrometry as a means to assess RBC deformability requires careful consideration of hematocrit and filter pore size. In general, it appears that the use of 5% hematocrit and five micron pores will lead to reliable results that are only slightly affected by small hematocrit errors when preparing a given suspension. However, it is recognized many experimental studies use small animal models and hence may have only small volumes of blood available for filtration studies; such studies may also use animals with small red blood cells (e.g., mouse, rat). In such situations the use of lower hematocrits and/or smaller pores may be appropriate, yet the use of 1 or 2% hematocrit suspensions is not without risks due to extreme sensitivity of RCTT to minor deviations of hematocrit (Fig. 3).

For higher sensitivity measurements it is recommended to use other methods in parallel (e.g., pore transit analysis, ektacytometry), providing information on red blood cell deformability and to approximate the microcirculatory effects, too.

Acknowledgements


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