Rheologic changes of hypothermic preserved red blood cells
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Chapter 2

Use of hydroxyethyl starch for inducing red blood cell aggregation

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Abstract

The aggregation of human RBC remains of biological and clinical interest. Replacement of plasma proteins by polymers to induce RBC aggregation may help to unravel the fundamentals of the aggregation process. Two theories exist to explain the RBC aggregation mechanism: a depletion and a bridging theory. RBC aggregation induced by hydroxyethyl starch (HES) polymers increases with polymer size, which suggests that aggregation is induced via the bridging theory. In the present study, the electrophoretic mobility (EPM) was measured to investigate RBC aggregation induced by 200-kDa HES polymers. In addition, we evaluated if these polymers were useful for demonstrating aggregation differences between RBCs from healthy and type-1 diabetes mellitus (T1DM) subjects. Our results demonstrate that the EPM values of RBCs in 200-kDa HES solutions were less negative than could be predicted by the viscosity of the suspension, supporting the bridging theory. Furthermore, aggregation analysis using the LORCA demonstrated that 200-kDa HES solution enhanced RBC aggregation of healthy and diabetic subjects in a similar manner as standard 500-kDa dextran solutions. In conclusion: our data supports the bridging mechanism underlying 200-kDa HES induced RBC aggregation. In addition, both polymers are useful for demonstrating cellular induced aggregation differences between RBCs from healthy and T1DM subjects.
2.1. Introduction

RBC aggregation is an important determinant of the flow behavior of blood. In regions with low shear rate, human RBCs form linear stacks of cells and multi-cellular aggregates that markedly enhance the blood viscosity and further reduce the blood flow rate.\(^1\) Under physiological conditions RBC aggregates are easily dispersed when the blood flow rate increases. However, under pathologic conditions stronger and larger aggregates may form that are more resistant to dispersion by the blood flow, therewith contributing to the occlusion of microvessels and hindering adequate tissue perfusion.\(^2,3\) Despite numerous studies, the physiological role of RBC aggregation still remains unclear. It has been demonstrated that different RBC aggregation tendencies can exist between healthy individuals.\(^4\) In addition, enhanced RBC aggregation is observed in a variety of diseases such as cardiovascular disease, diabetes mellitus, renal failure, sepsis, thalassemia and sickle cell disease.\(^3,5,6\)

The RBC surface consists of a layer of proteins, called the glycocalyx, which bears a net negative charge. The ability of RBCs to aggregate is a balance between repulsive and attractive forces. On one hand RBC aggregates are dispersed by repulsive electrostatic forces between negatively charged RBCs as well as the prevailing shear forces generated by the blood flow. On the other hand the promotion of aggregation is induced by the presence of plasma proteins, especially fibrinogen, or by high molecular weight polymers.\(^5,7\) Usage of high molecular weight polymers to induce RBC aggregation gives more insight in the aggregation process because it will exclude aggregation differences that are induced by variations in plasma protein composition. Non-ionic polymers like dextran, polyethylene glycol and to a lesser extent hydroxyethyl starch (HES) have been investigated for their RBC aggregation inducing properties.\(^8,14\) Like dextran, HES polymers are used in plasma substitutes to treat for example hypovolemia during surgery.\(^15,16\) HES polymers are available in a broad range of molecular weights all with different tendencies to promote RBC aggregation. In this regard, only HES polymers with low aggregation tendency, such as the 130-kDa HES solutions, are currently preferred for infusion, whereas HES polymers with hyper-aggregating tendencies, such as 450-kDa HES solutions, are less favored in clinical practice.\(^11,17,18\) However, the 200-kDa HES polymer, which has intermediate RBC aggregation tendencies, could be useful as a pro-aggregant in RBC rheologic studies.
At present there are two theories that explain the RBC aggregation mechanism induced by high molecular weight polymers. In the bridging theory, it is hypothesized that macromolecules adsorbed onto the RBC membrane form bridges between adjacent cell surfaces, which help overcome repulsive electrostatic forces between the RBCs. In the depletion theory, RBC aggregation occurs due to a lower concentration of macromolecules near the RBC surface compared to the suspending medium. This depletion of macromolecules near the cell surface leads to an osmotic gradient that causes fluid to move away from adjacent RBCs, which subsequently favors RBC aggregation.

Previous data support the depletion theory as the mechanism of dextran induced RBC aggregation. The mechanism through which HES induces RBC aggregation has not yet been established. However, since stronger and larger RBC aggregates are formed with HES solutions of increasing molecular weight, an adsorption mediated aggregation mechanism is expected to exist for this polymer. This is in contrast with the depletion mechanism, where RBC aggregation has a nonlinear dependency on the molecular weight of the polymer.

Electrophoresis has shown to be a useful tool for studying the aggregation behavior of RBCs. By measuring the electrophoretic mobility (EPM) of RBCs in polymer solutions, a better understanding of the underlying aggregation mechanism can be obtained. The EPM of RBCs in saline solutions demonstrates a negative value. In this regard a less negative EPM of RBCs in polymer solutions (i.e. a lower mobility of RBCs) suggests bridging mediated aggregation due to interactions of polymers with the RBC surface. In contrast, a more negative EPM value (i.e. a higher mobility of RBCs) is explained by depletion of polymers near the RBC surface.

Differentiating between plasmatic and cellular factors leading to altered RBC aggregation is important from both a biological and clinical point of view. Usage of high molecular weight polymers will manifest RBC aggregation differences which are induced by cellular factors such as RBC shape, deformability and membrane surface properties. This intrinsic tendency of RBC to aggregate (i.e. RBC aggregability) is dependent on the type and the molecular weight of polymers in solution.

In the past standard dextran polymers have been useful pro-aggregants in RBC rheologic studies. Like the standard 500-kDa dextran polymers, the 200-kDa HES polymers could be useful to manifest cellular induced aggregation differences between RBCs from healthy individuals and patients with a disease. Blood from type-1 diabetes mellitus (T1DM)
patients is especially suitable for aggregation testing. Particularly, because in these patients alterations in both plasma and RBC cellular properties are responsible for enhanced RBC aggregation tendencies. Nowadays, RBC aggregation can be studied using the LORCA. This technique allows RBC aggregation to be studied ex vivo either in whole blood or in polymer solutions.

In this study, the EPM of RBCs from healthy volunteers was explored in 200-kDa HES and standard 500-kDa dextran solutions in order to elucidate the underlying aggregation mechanism. Furthermore, the LORCA was used to investigate if these polymers could discriminate cellular induced aggregation differences between RBCs from healthy volunteers and T1DM patients.

2.2. Materials and methods

Sample preparation

Blood was collected from healthy volunteers and T1DM patients after informed consent and in accordance with University Medical Center Groningen protocols. Briefly, whole blood was anticoagulated with EDTA (1.5 mg/ml) and part of it was used in aggregation experiments within 1 hour after collection. To obtain RBCs, whole blood was centrifuged at 1100 x g for 12 min to remove the buffycoat and supernatant. The concentrated RBCs were washed twice with PBS (pH 7.4), and resuspended in 0.9% sodium chloride solution (NaCl; pH 4.5 or pH 7.4) to a final hematocrit (Hct) of 40 ± 1%. Aggregation experiments and EPM measurements with washed RBCs were performed within 4 h after collection. A 10% HES solution with a molecular weight of 200-kDa and a molar substitution of 0.5 was obtained from Fresenius, 's-Hertogenbosch, the Netherlands. Dextran fractions with a molecular weight of 500-kDa were obtained from Sigma-Aldrich, Steinheim, Germany. A 2% dextran solution (wt/vol) was prepared by dissolving this polymer into 0.9% NaCl solution with pH 7.4.

Electrophoretic mobility and viscosity measurements

The speed of RBCs in an electrical field was determined in suspension using a Laser Zee Meter 501 equipped with image analysis options (PenKem, Bed ford Hills, N.Y.), as has been described previously. Briefly, RBCs were suspended in either 0.9% NaCl pH 7.4,
0.9% NaCl pH 4.5, 10% 200-kDa HES pH 4.5 or 2% 500-kDa dextran pH 7.4 solutions at a Hct of 0.05%. The electrophoresis chamber was filled with the RBC suspension and a voltage difference of 150V was applied over the chamber. EPM values were measured at 20°C in duplicate with RBCs obtained from four different healthy volunteers. EPM values were obtained from samples that contained a minimum of 35 RBCs per suspending solution. The viscosities of the suspending solutions were measured in duplicate at 20°C using a viscometer (DV-II+ Pro, Brookfield, USA). All EPM values are reported as measured and are not corrected for differences in viscosity between the various solutions.

**Aggregation measurements**

RBC aggregation was assessed in vitro, by a laser-assisted optical rotational cell analyzer (LORCA; R&R Mechatronics, Zwaag, the Netherlands). Aggregation was tested with 1 ml of either whole blood or with RBCs suspended in 200-kDa HES or 500-kDa dextran solutions. In short, RBC suspensions were centrifuged for 1 min at 3500 x g and the supernatant was discarded. RBCs were resuspended in either 10% 200-kDa HES or 2% 500-kDa dextran solutions, to obtain a Hct between 35 ± 5%. The aggregation of the RBCs was monitored after complete disaggregation under increased shear stresses. Both the aggregation measuring procedure and the subsequent analyses were computer controlled. Aggregation of RBCs was expressed by the aggregation index (AI), where a larger AI reflects an increased ability to aggregate. The AI depends on the kinetics and extent of aggregation. The AI was determined after correcting the Hct in all the samples to a constant value of 45%. The kinetics of aggregation (T_{1/2}) was expressed by the time necessary to induce 50% aggregation. All measurements were performed at 37°C.

**Statistical analysis**

Statistical analysis was performed using statistical software (SPSS, version 16.0, SPSS Inc., Chicago, IL). Data were tested for normality with the Kolmgorov-Smirnov goodness-of-fit test. The EPM values were analyzed by using a repeated-measure ANOVA. Post hoc comparisons were performed to quantify differences between suspension solutions by using paired t-tests.

Aggregation differences between RBCs from healthy and T1DM subjects were determined by using unpaired t-tests. Within subjects, paired t-tests were performed to show
Polymer induced RBC aggregation

A Bonferroni correction was applied to correct for multiple comparisons. Differences are considered to be significant with a two-tailed p-value of less than 0.05. Results are presented as means ± SD.

2.3. Results

RBC electrophoretic mobility and viscosity

The EPM of RBCs suspended in polymer-free saline solutions was not significantly affected by the pH. RBCs suspended in 200-kDa HES solutions showed significantly less negative EPM values as compared to RBCs suspended in polymer-free solutions (-0.04 vs -0.58 \(10^{-8}\) m\(^2\)V\(^{-1}\)s\(^{-1}\)). However, the EPM values of RBCs suspended in 200-kDa HES solutions were less negative, than would be expected based upon the inverse relation between suspension medium viscosity and EPM values found (Figure 2.1A).\(^{28}\)

In contrast, RBCs suspended in 500-kDa dextran solutions demonstrated significantly more negative EPM values as compared to RBCs suspended in polymer-free solutions (-1.40 vs -0.74 \(10^{-8}\) m\(^2\)V\(^{-1}\)s\(^{-1}\)). However, these observed EPM values were considerable more negative than would be expected based upon the inverse relation between the suspending medium viscosity and the EPM values found (Figure 2.1B).

RBC aggregation

Whole blood from T1DM subjects showed an increased tendency to aggregate as compared to that of healthy subjects (p < 0.05: Figure 2A). Moreover, the AI induced by 200-kDa HES solution is higher with RBCs from T1DM subjects than that of healthy subjects (p < 0.05). However, it was notable that the AI induced by 200-kDa HES polymers was similar to the AI induced by the 500-kDa dextran polymers.

No significant differences in the aggregation half-time between whole blood from healthy and T1DM subjects (Figure 2B) were observed. The 200-kDa HES solution markedly lowered the T\(_{1/2}\) as compared to that observed with whole blood of healthy and T1DM subjects (p < 0.05), indicating a faster aggregation process with the use of this polymer. However, both polymers did not induce significant differences in T\(_{1/2}\) between RBCs from healthy and T1DM subjects.
Figure 2.1. The electrophoretic mobility of human RBCs suspended in 200-kDa HES (A) or standard 500-kDa dextran (B) solutions as a function of their viscosity. The dashed line is the predicted relation between EPM and suspension viscosity assuming an inverse relation between these variables. Values are expressed as mean of eight experiments.
Figure 2.2. Aggregation indexes (A) and aggregation half-time (B) for RBCs obtained from healthy and T1DM human subjects in either autologous plasma or RBCs resuspended in polymer solutions. Values are expressed as the mean ± SD of ten different subjects. Significant changes are illustrated in the figure (p < 0.05); * significantly different from whole blood; † significantly different from its healthy counterpart.
2.4. Discussion

The found EPM values of healthy RBCs suspended in 200-kDa HES solution supports the bridging theory as the mechanism underlying HES induced RBC aggregation. Although 200-kDa HES and 500-kDa dextran polymers induce RBC aggregation via different mechanisms, we demonstrated that both polymers are useful pro-aggregants for manifesting cellular induced aggregation differences between RBCs from healthy and T1DM subjects.

Aggregation of RBCs is a physiological phenomenon that has been studied for decades. Although RBC aggregation is an important determinant of blood viscosity in regions of low shear rate, the physiological role of this process still remains elusive. Nevertheless, enhanced RBC aggregation has been observed in various diseases. The use of standard polymer suspensions to induce RBC aggregation will give more insight in the fundamentals of the aggregation process. Currently, HES polymers are primarily used as plasma expanders. However, studies demonstrating that 200-kDa HES polymers could be useful pro-aggregants in rheologic studies were lacking.

In the present study, the EPM values of RBCs suspended in 200-kDa HES solution were considerably less negative than the linear reciprocal viscosity change that is predicted by the Helmholtz–Smoluchowski equation. The EPM of RBCs in an electrical field is determined by the viscosity of the suspending fluid as well as the RBC surface charge density and the glycocalyx thickness. Given that HES polymers are nonionic, it is unlikely that these EPM changes are associated with alterations in RBC surface charge density. Instead, absorption of nonionic HES polymers onto the RBC surface may have attributed to structural changes within and near the RBC glycocalyx, explaining the less negative EPM values. Clearly, our EPM measurements underline the presence of a bridging mechanism between RBCs when using 200-kDa HES polymers.

In contrast, dextran induced aggregation is characterized by an optimal aggregation tendency at a molecular weight of approximately 500-kDa. In this study, the EPM values of RBC in 500-kDa dextran solutions became more negative despite the higher viscosity of the bulk suspending medium. These findings are in agreement with a depletion interaction in which a lower viscosity near the RBC surface compared to the bulk suspending medium, enhances the EPM of the RBCs. A drawback with current depletion theories is that polymer absorption onto RBC membranes are often not taken into account due to experimental limitations. Nevertheless, a depletion theory does not require an absolute
lack of absorption on RBCs in order to initiate aggregation, but merely that the absorbed polymer level is less than the polymers present in the bulk suspending medium.\textsuperscript{5,31} Recently, Liu \textit{et al.} have provided new insights for characterizing absorption of polymers onto RBCs.\textsuperscript{32} In their study tritium labeling was used to quantify the in vitro absorption of polymers onto the RBCs, while laser scanning confocal microscopy was used to locate the precise binding sites. Future aggregation studies that allow the implementation of an absorption interaction with these techniques are therefore warranted.

In the present study, whole blood from T1DM human subjects showed an increased tendency to aggregate as opposed to whole blood from healthy subjects. It has been recognized that a disturbed glucose homeostasis in T1DM subjects results in elevated plasma fibrinogen concentrations and a reduced anionic RBC surface charge.\textsuperscript{25,33} The increased AI observed with whole blood from T1DM subjects is thus a result of changes in both plasma and RBC cellular properties.

Usage of high molecular weight polymers, exclude aggregation differences induced by variations in plasma protein compositions. In this study, the 200-kDa HES and standard 500-kDa dextran solutions markedly enhanced the AI of RBCs as compared to whole blood. Nevertheless, both polymer solutions were clearly able to discriminate aggregation differences between RBCs from healthy and T1DM subjects. Our results therefore demonstrate that also 200-kDa HES polymers are useful in manifesting RBC aggregation differences which are of cellular origin. The parameter AI depends both on the kinetics (t\textsubscript{1/2}) and extent of aggregation. In this study no significant differences in t\textsubscript{1/2} between healthy and T1DM subjects with either whole blood or polymer induced RBC aggregation could be observed. This indicated that aggregates are not necessarily formed faster but that the extent of aggregation is the determining factor when it comes to manifesting differences between RBCs from healthy and T1DM human subjects.

The ability of RBCs to form aggregates in the presence of plasma proteins may play a pivotal role in maintaining hemodynamics. RBCs flow from the endothelial wall into the center of the blood vessel where they form aggregates. While physiological values of RBC aggregation direct leukocytes and possibly platelets from the axial core towards the vascular wall,\textsuperscript{34} intensified RBC aggregation may hinder or obstruct the blood flow in micro-vessels. More insight in the RBC aggregation process is therefore important from both a biological and clinical point of view.
In the past, the depletion theory has been particularly favored as the mechanism of non-ionic polymer induced RBC aggregation. Although the 200-kDa HES solutions induced similar aggregation tendencies as the standard 500-kDa dextran solutions, our data supports the bridging theory as the mechanism underlying HES induced aggregation. Since RBCs from healthy volunteers show significant variations in terms of their aggregation tendency, it may be in vivo both aggregation mechanisms can exist depending on the protein composition of plasma. Furthermore, our results demonstrate that like the standard 500-kDa dextran solutions, the 200-kDa HES solutions are useful for manifesting cellular induced aggregation differences between RBCs from healthy and T1DM subjects.

References


