HEMORHEOLOGICAL PARAMETERS: PROGNOSTIC VALUE AND MEASUERMENT

Ph.D. thesis

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I. HEMATOKRIT/BLOOD VISCOSITY RATIO: PROGNOSTIC VALUE AND USE IN THE ASSESSMENT OF THE OPTIMAL HEMATOCRIT

Introduction

Cardiovascular diseases are the leading cause of morbidity and mortality in the developed countries. „Classical" risk factors like diabetes, hypertension, dyslipidemia, smoking and obesity are long known. Researches suggest other factors in the pathogenesis as well. The importance of hemorheological factors was discovered in the past two decades. Hemorheological parameters describe the flow properties of blood and the physical properties of the blood cells. Impairment of these factors may lead to damage of microcirculation and the increase of the work of the heart. Best known is the role of hematokrit, plasma and whole blood viscosity and fibrinogen, they are the most widely used parameters in clinical practice as well.

In case of anemia or low erythrocyte count, blood cannot bind and deliver enough oxygen, decreased hematokrit is associated with increased cardiac mortality. Increase in hematocrit leads to a linear increase in oxygen binding capacity and an exponential increase in blood viscosity. Though blood becomes richer in oxygen, increased flow resistance, slowing of flow cause a net decrease in oxygen supply. A much investigated problem of hemorheology is which hematocrit would be optimal. Oxygen delivery intensity depends on the blood flow intensity and the concentration of oxygen binding hemoglobin. The former can be described by the Hagen-Poiseuille law, the latter can be approximated by hematocrit. If flow geometry and pressure gradient is constant, oxygen delivery intensity depends on hematocrit/blood viscosity ratio.

$$Q_{O_2} \approx c\, Q_{blood}\, Hb \approx c\, \frac{\Delta P\, \pi\, r^4}{8\, \eta\, l} Hct = C\, \frac{Hct}{\eta}$$

If cardiovascular diseases are related to the impairment of perfusion, hematocrit/blood viscosity ratio may probably be a stronger predictor than the former parameters. Despite, few studies concern this topic. We investigated the clinical usefulness on hematocrit/blood viscosity ratio.

Aims

We aimed to investigate the relationship between hematocrit/blood viscosity ratio and cardiac mortality and morbidity.

Determination of hematocrit/blood viscosity curve needs much measurements, time and blood samples. We seeked a faster and simpler method to determine it for clinical use. As a part, we aimed to determine the maximal point of the curve, the virtual optimal hematocrit.

We aimed to investigate the relationship between this theoretic virtual optimal hematocrit and the empiric data of clinical patients.

1. Low hematocrit/blood viscosity ratio as a mortality risk factor in coronary heart disease

Patients and methods

68 patients with coronary heart disease were referred to elective coronary angiography at our institution in 1996-1997. Coronary angiography was performed from femoral arterial access according to the standard Judkins technique and neither stent insertion nor dilatation was performed. The extension of main vessels' narrowing was scored 0 to 4 resulting in a maximum of 12 for all coronaries. Before intervention blood samples were collected to determine total cholesterol and triglyceride levels, HDL/LDL cholesterol ratio, fibrinogen level, hematocrit (with capillary tube centrifuge), plasma (PV) and whole blood viscosity (BV) at 90 s^{-1} at 37 ºC (Hevimet 40 capillary viscometer). Left ventricular ejection fraction and cardiac index was measured by echocardiography and impedance cardiography, respectively. Besides, we registered gender, age, body mass index, history of smoking, hypertension, diabetes and past myocardial infarction.

In February 2006, the local council registry was inquired in order to identify patients who had died during the follow-up period. A questionnaire was mailed to all patients alive to obtain information on the number of hospitalizations due to cardiac problems. For patients who had died during follow up, the family physicians were contacted in order to obtain the cause of death.

Patients who died due to cardiac cause (n=10) during the follow up were classified as group C while others who died due to non-cardiac cause (n=2) or were alive (n=66) as group NC. Pearson Chi-square or Fisher's exact tests as appropriate were performed to compare dichotomous characteristics, while Student t-tests were performed in case of continuous variables. Receiver operating characteristic (ROC) curve and the area under the respective curve (AUC) was calculated in order to determine predictive powers of parameters for cardiac mortality. According to each parameter, patients were divided into upper und lower halves, and Kaplan-Meier survival analyses were performed to assess the impact of respective parameters on survival. Non-cardiac deaths were declared censored. In case of survivors linear bivariate correlation coefficients were calculated to see how well parameters predict the severity of coronary heart disease, which was represented by the number of hospital admissions due to cardiac problems. Statistical analyses were performed using the SPSS 11.0 software.

Results

Group C patients were significantly older (62.3±10.1 vs. 53.3±8.9 years), had lower triglyceride level (1.8±0.5 vs 2.6±1.6 mmol/l) and Hct/BV ratio (87±5 vs 93±9 $Pa^{-1}s^{-1}$) than group NC patients. Considering the ROC curves, conventional parameters failed to predict cardiac mortality, while the Hct/BV ratio was able to do it (AUC: 0.716, $p = 0.028$). Best cut-off value for Hct/BV was found at 88.8 $Pa^{-1}s^{-1}$ (sensitivity 70%, specificity 70%). Kaplan-Meier analysis demonstrated significant difference between the upper and lower halves according to the respective parameters only in case of fibrinogen ($p = 0.030$) and the Hct/BV ratio ($p = 0.009$). Thirty-four patients provided information via the questionnaire about their hospitalization. None of the examined parameters (age, BMI, triglyceride, total cholesterol, HDL/LDL ratio, fibrinogen, Hct, PV, BV, Hct/BV ratio, cardiac index, left ventricular ejection fraction) showed correlation with the number of hospital admissions. However, after

excluding one obvious outlier the Hct/BV ratio resulted in a significant linear correlation (Pearson r = -0.377, p = 0.03).

Discussion

Our finding on the role of fibrinogen matches the literature data. In our study, however, we failed to demonstrate any significant predictive power of many conventional cardiovascular risk factors for mortality. This might have been due to the fact that we studied a relatively severely diseased population. We can assume that the conventional risk factors had influenced our patients' health state mostly before the start of follow up period and probably their further impact was less pronounced. Moreover, we selected mainly momentary parameters, and had information neither about previous abnormalities, which could have been masked by the therapy our patient already had received, nor about the dynamic of these values during the follow up period. On the other hand, upon comparing group C and NC, most risk factors showed the anticipated tendency only without reaching the level of significance, possibly due to the relatively small sample size. Similarly, correlation of risk factors with number of hospitalization mostly matched the expected tendency. For example, both high (above 44%) and low (below 36%) levels of Hct was associated with frequent hospitalization, while less hospital admissions could be seen at moderate levels of Hct. After performing a multivariate regression analysis, none of the mentioned parameters were found to be an independent risk factor for hospitalization. The value of this analysis is, however, limited. Firstly, the sample size was rather small, and was even lowered due to missing values. Secondly, considerable interference was present between variables. Such interferences are well known between BMI and lipid parameters, age and fibrinogen, lipid values and PV, fibrinogen and PV, Hct and BV or age and cardiac index.

Our study has some further limitations. A decade ago the availability of coronary angiography was much more limited than nowadays, so usually only the most severe cases were elected for it. More than half of the studied patients had severe three-vessel-disease. Such biased distribution prevented us from reporting any statistical analysis including coronary angiography score as a direct variable. When assessing survival, we only considered the hard endpoint cardiac death, and ignored other follow up information like a later coronary angiography and stent implantation, or coronary revascularization, although such interventions probably affect survival.

In summary, the novel finding of this study is the increased cardiac mortality risk and morbidity of CHD patients with unfavorable Hct/BV ratio. Hct is a frequent, while BV is a rare investigation in CHD. However, also their quotient could be taken into consideration when cardiac risk is estimated. This suggestion warrants further larger and prospective follow up studies in the future.

2. New method to determine hematocrit/blood viscosity ratio and virtual optimal hematocrit

Introduction

Blood viscosity is exponentially related to hematocrit according to Mátrai, thus the relationship between hematocrit and hematocrit/blood viscosity ratio can be described by an

x/e^x type function. Its local maximum point denotes to the maximal oxygen delivery intensity at the optimal hematocrit value.

Blood is a suspension of cells in plasma, its viscosity is strongly influenced by plasma viscosity. Blood is a non-Newtonian fluid, its viscosity depends on the shear rate. At low shear rate viscosity steeply increases primarily because of erythrocyte aggregation. At high shear rates blood viscosity converges to a limit partially influenced by erythrocyte deformability. Our workgroup generally uses blood viscosity measured at 90 s^{-1}, where the former factors are considered balanced. Hct/BV curves and optimal hematocrit values depend on shear rate likewise. The set of curves at various shear rates is called Hct/BV profile.

We aimed to make a method to determine Hct/BV profile and optimal hematocrits in a simple way.

Methods and samples

Heparin anticoagulated blood samples of 7 volunteers were used. Besides control fraction, fractions with hematocrit values of 10, 25, 40, 55 and 70% were prepared. Hematocrit was determined with capillary tube centrifuge. Plasma and whole blood viscosity was measured with Hevimet 40 (Hemorex Ltd, Budapest) capillary viscometer that describes the relationship between shear rate and viscosity by the Casson's principle. Using the parameters of the control fraction, blood viscosity expected at 10, 25, 40, 55 and 70% hematocrit was calculated using Matrai's formula as well.

Results and discussion

Comparison of measured and calculated viscosities of various hematocrit samples led to the following findings. In samples with hematocrit of 70% calculated viscosity is significantly lower than measured at shear rate of 50 s^{-1} and above. This tendency is visible even at 30 s^{-1}. At 10 s^{-1} the difference is not significant, but standard deviation is very high at any hematocrit. This can be seen also at 30 s^{-1} in a milder form.

As a result of the operaton principle of the capillary viscometer, high viscosity 70% hematocrit samples are actually measured in a shear rate range of 10-100 s^{-1}, situated at the steep arm of the Casson curve, where points have a considerable stochastic error. When extrapolating to high shear rates and calculating the asymptote this error is exaggerated. Similarly, if points mainly reside on the flat, asymptotic arm (e.g. Hct of 40%, shear rate of 40-240 s^{-1}, extrapolation to low shear rate points on then steep arm will be prone to error. Using a rotational viscometer, in which shear rate can be controlled better, these errors would probably be smaller.

It is proven that Casson equation cannot describe blood viscosity on the entire shear rate range with only a single constant pair (Casson viscosity and yield shear stress). Hematokrit-blood viscosity relationship is also more complicated than an exponential function (it is influenced by the changing erythrocyte aggregation and shape according to shear rate, ratio of the intracellular and the suspending plasma viscosity). Because of this inadequacy, calculated and measured viscosities are equal only at the hematocrit of the control fraction (ca. 40-50% Hct) and at 0%, and increasingly differ at more distant hematocrits.

Our results show that measured viscosity of samples with hematocrit of 10 to ca. 60% at shear rates above 10 s^{-1} are within ±10% difference from calculated viscosity at 95% confidence level. Such inaccuracy is probably not biologically significant either. The two

methods give an acceptable agreement in the clinically relevant hematocrit and shear rate range. Results are similar for Hct/BV with the opposite sign.

Only points with predefined hematocrits can be measured on the Hct/BV curve. Thus, optimal hematocrit cannot be measured, merely estimated between which two predefined hematocrits it resides. Setting the hematocrit is time consuming. It is possible to refine the curve by increasing the number of measurement points, but time and sample needed increases proportionally.

Contrarily, the whole Hct/BV profile can be calculated from a single measurement. No extra preparation or hematocrit setting is required, needing considerably less time and blood. The hematocrit, blood and plasma viscosity values of routinous clinical measurements are eligible to draw the Hct/BV profile. A disadvantage of this method is that the Hct/BV profile depends on the accuracy of a single measurement. In case of a measured Hct/BV curve, the fractions serve as reference for each other, in case of calculation this inner control is missing.

Hct/BV values can be calculated for any Hct with a closed formula that allows to determine its local maximum point using differential calculus:

$$Hct_{opt} = \frac{Hct_0}{ln\frac{BV_0}{PV}}$$

(Hct_0, BV_0: hematocrit and viscosity of baseline sample)

Keep in mind, however, that Hct/BV curve and the optimal hematocrit is only valid in controlled Hagen-Poiseuille flow and predefined shear rates, in vitro. Do not confuse it with the holistic (or at least situative), clinically relevant optimal hematocrit, in vivo. To emphasize this difference, the previously described parameter will be referred to as virtual optimal hematocrit ($Hct_{opt,v}$).

3. Relationship of virtual and global optimal hematocrit in patients with acute coronary syndrome

Introduction

Definition and calculation of virtual optimal hematocrit ($Hct_{opt,v}$) was introduced previously. The clinically relevant, global optimal hematocrit ($Hct_{opt,g}$) is harder to determine. It can refer to either the lowest mortality, the best quality of life, the maximal sport performance or the best perfusion of an area with critical ischemia. In either approach, blood flow is investigated in a complex in vivo system, where conditions of Hages-Poiseuille flow are not fulfilled and shear rates change dynamically. Hct/BV model has a limited relevancy among such conditions.

We investigated the relationship of virtual and global optimal hematocrits.

Patients and methods

In our retrospective study data of 122 acute coronary syndrome patient (57% male, mean age: 50±12 years) was used. Hemorheological measurements were performed on the day of admittance to hospital, 2 and 6 days later, and during the 6 and 12 month follow-up visits, respectively. Hematocrit, plasma and whole blood viscosity was determined. $Hct_{opt,v}$ was calculated as described before. $Hct_{opt,g}$ could not be measured objectively. As

theoretically homeostasis aims to maintain optimal conditions when undisturbed – though accepting its limited relevancy – we defined global optimal hematocrit equal to empirical hematocrit.

Results and discussion:

We investigated correlations between $Hct_{opt,g}$ and $Hct_{opt,v}$ calculated at various shear rates. Correlations proved to be stronger at high shear rates. Circulatory system consists of several vessel regions with various shear rates. The same blood flows within them, though their $Hct_{opt,v}$ would be different. $Hct_{opt,g}$ is a compromise of these values. Vessels with greater hemodynamic significance, like high resistance arterioles and capillaries, probably have a higher emphasis on its formation. Shear rates in aforementioned vessels range from several hundred to several thousand s^{-1}. Strongest correlations were found at such shear rates.

Correlations during the hospital phase were weaker. During the treatment of acute coronary syndrome patients receive infusions that cause hemodilution. Relationship between empiric hematokrit and $Hct_{opt,g}$ aimed by the homeostasis weakens, and thus weaken all correlations based on the equality of the two parameters. In the follow-up period (6 and 12 month values) no such outer disturbance is present, correlations are stronger.

Though empiric hematocrit decreased during the hospital phase, $Hct_{opt,v}$ values remained practically unchanged. Constancy of $Hct_{opt,v}$ values, however, does not necessarily mean the constancy of $Hct_{opt,g}$. When hemodynamic conditions and shear rates change, emphasis of various $Hct_{opt,v}$ values change and the compromise value shifts as well. In acute coronary syndrome a circulatory redistribution is present due to the decrease in cardiac output. For the optimal state, change of hemorheological conditions should serve the perfusion of lower resistance central regions as well. Immobilization of patients leads to the decrease of blood flow velocity and shear rates as well. As lower shear rates is accompanied by lower $Hct_{opt,v}$ values (lower shear rate leads to higher blood viscosity, the denominator ln(BV/PV) in the formula of $Hct_{opt,v}$ increases, thus $Hct_{opt,v}$ decreases), $Hct_{opt,g}$ probably decreases as well.

Males are long known to have higher hematocrit than females, most presumably because of females' regular blood loss due to menstruation. We have found no difference in $Hct_{opt,v}$. It is possible that there is really no difference in $Hct_{opt,v}$ for the two sexes, and the empirical difference is an evolutional consequence (males, who were more aggressive and in precivilized eras more prone to injuries, may have a larger blood reserve). On the other hand, $Hct_{opt,g}$ of males may be higher than of females even with similar $Hct_{opt,v}$ values, if higher shear rates are dominant in males.

We found significant positive correlation between plasma viscosity and $Hct_{opt,v}$ values. As $Hct_{opt,v}=Hct/ln(BV/PV)$, increase in PV leads to decrease in $Hct_{opt,v}$, thus the mentioned correlation seems to be a mathematical necessity and not meaning a biologically valid relationship. We may handle correlation between $Hct_{opt,v}$ and empiric hematocrit similarly. The present study cannot adequately identify global optimal hematocrit and validate the aforementioned correlations. However, if it may be true that at higher plasma viscosity $Hct_{opt,v}$ is higher, and at higher $Hct_{opt,v}$ leads to higher $Hct_{opt,g}$ and empiric hematocrit, then we may expect higher empiric hematocrit at higher plasma viscosity. In our study these two parameters showed significant correlation. As plasma viscosity and hematocrit are two directly measured parameters, this correlation exists independently from us. The mentioned theory may be an explanation, but still not necessarily valid. Plasma viscosity and hematokrit

may be influenced by a common factor in the background (e.g. smoking) without the need for $Hct_{opt,v}$ as a logical connector. Further studies are needed to prove or deny the theory.

II. METHODOLOGICAL PROBLEMS WITH MEASUREMENT AND INTERPRETATION OF HEMORHEOLOGICAL PARAMETERS

4. The impact of sample preparation on measured hemorheological parameters

Introduction

Blood sampling and sample procession often take place at different location. Transportation and limited processing capacity lead to delay before measurement, while blood is stored out of its original environment. Properties of blood component may change among the aphysiological conditions, and parameters describing them may show a false picture. We seeked, how long storage time has a yet negligible impact on measurement of hemorheological parameters.

For some measurements samples must be taken through aphysiological preparation processes. Centrifugation is often required, where cells are subjected to several thousand g acceleration. When measuring erythrocyte filterability or aggregability (i.e. aggregation among standardized circumstances), cells are washed and suspended in media very different from plasma. We studied the impact of such preparation methods.

Changes of erythrocyte deformability

Erythrocyte deformability was measured with LORCA (Laser-assisted Optical Rotational Cell Analyzer, Mechatronics BV, The Netherlands), and characterized by elongation index (EI) at shear rates between 0.30 to 30 Pa (see detailed operation principle in next chapter)

Elongation index did not change significantly at any shear stresses after 1 or 2 hours of storage at room temperature. After 3 hours, however, EI showed a significant decrease when measured at 1.69 Pa or above. After 6 hours this change became more pronounced and the decrease in EI at 0.95 Pa reached significance as well. EI did not change at any shear stress if the sample was stored at 4ºC for 1 day. After 2 days of storage a tendency was visible: at lower shear rates (below 9.49 Pa) EI tended to increase, at higher shear rates (above 9.49 Pa), however, it decreased. After 1 week of storage this tendency became significant.

Erythrocyte deformability is considered to be determined by three main factors. If other parameters are unchanged, increase of *internal viscosity*, and the *stiffening of membrane* decrease deformability, while the increase of *surface to volume ratio* improves it. During storage, however, these factors change simultaneously, and the magnitude of these changes develops differently in time. Moreover, the importance of each factor is probably different on the deformation at different shear stresses.

Literature data suggest several mechanisms in the background of changes:

- Energy depletion, loss of potassium and water (increase of inner viscosity and increase of surface to volume ratio).
- Increase of membrane stiffness due to lactic acid accumulation.
- Change in membrane composition: removal of sialic acid, rearrangement and phase separation of phospholipids, degradation of membrane and cytoskeletal proteins, echinocyte formation, shedding of vesicules.
- Alteration of immunogenity, damage from immune mechanisms.

To assess if mechanical stress during centrifugation affects deformability, samples were centrifuged at 2500 g for 10 minutes and remixed. We found no change in deformation even after centrifugation 3 times.

To assess washing with buffer, samples were centrifuged at 2500 g for 10 minutes, plasma and buffy coat were removed, and red cells were resuspended in phosphate buffered saline (PBS), this procedure was done 3 times. We found the significant decrease of EI above 0.95 Pa. The aforementioned results suggest that the impact of centrifugation and the past short time is negligible. A minor difference between medium and intracellular osmolarity would not cause difference either, as final osmotic equilibrium develops in the suspending medium used for measurement. We suspect that repeated washing with buffer removes plasma proteins, and proteins loosely attached to erythrocyte membrane as well, that may alter viscoelastic properties of the cell membrane. Contrary to our findings, washing of stored blood before transfusion was reported to cause no additional change of deformability in the literature.

Changes of erythrocyte aggregation

We used two instruments for measurement.

In LORCA whole blood sample is disaggregated at 500 s^{-1} shear rate in Couette-flow, then released. Red laser light backscattering intensity is measured for 120 seconds. Aggregation index (AI) is the integral of backscattering intensity change during the first 10 seconds corrected to the possible maximal change. Threshold shear rate is the lowest shear rate that can maintain complete disaggregation.

In Myrenne MA-1 aggregometer (Myrenne GmBH, Germany) whole blood sample is disaggregated at 600 s^{-1} shear rate. In M mode shear is stopped, while in M1 mode a further 3 s^{-1} shear is maintained. Aggregation indices represent the uncorrected integrals of intensity change of transmitted infrared light during the first 10 seconds.

Neither aggregation index nor threshold shear rate of LORCA changed significantly after 1, 2 or 6 hours of storage at room temperature. Storage at 4ºC for 1, 2 or 7 days caused an unpredictable fluctuation in AI: though the mean of values did not change significantly, their variance increased. Threshold shear rate, on the other hand, decreased significantly. Both Myrenne M and M1 indices decreased significantly already after 2 hours of storage, and decreased even further afterwards.

Fibrinogen degradation and stomatocyte formation decrease aggregation. During long-term storage the diminishing of cell surface negative charge and formation of echynocytes increase aggregation. In rats, an initial decrease in aggregation was followed by a later increase. In larger body weight organisms, this phenomenon may be slower, and we may have seen only its beginning in our study on humans.

Correlation between LORCA and Myrenne was surprisingly weak. We suggest the following as a reason: 1, LORCA AI may be less influenced by initial optical properties (e.g. change in light absorbance spectrum, free hemoglobin, sample hematocrit) due to the correction of the index. 2, In LORCA measurement is performed at 37ºC, while in Myrenne at room temperature; temperature influences aggregation and deformability as well. 3, In LORCA light backscattering, in Myrenne light transmission is recorded.

Changes during storage is reported to happen more rapidly if erythrocytes are left packed together than kept mixed in plasma; probably due to the faster consumption of local glucose and faster accumulation of acidic metabolites in the smaller compartment. To assess the phenomenon, some samples were being kept on a rollerbank for 2, 4, and 6 hours instead of standing still. In case of LORCA, we found no significant change. In case of Myrenne, even more pronounced changes were found at rolled samples. We suspect that continuous rolling gives erythrocytes an increased probability to meat the tube wall, which may be more important than the formation of a different metabolic environment. We used polypropylene tubes, another material like glass may have given different results.

Changes of viscosity

For plasma and whole blood viscosity measurements we used a Hevimet 40 (Hemorex Ltd., Hungary) capillary viscometer. Measurements were performed at 37°C. Hematocrit was determined parallel using a capillary hematocrit centrifuge (Heraeus Instruments, Germany).

When blood was stored at room temperature, neither low (10 s^{-1}), mid (90 s^{-1}) or high (240 s^{-1}) shear blood viscosity changed significantly during 1, 2, 3 or 6 hours. The same was true for plasma viscosity, Casson viscosity and yield shear stress as well. The confidence interval of change was broad for 10 s^{-1} blood viscosity and Casson viscosity both in absolute and relative manner. The same was true for yield shear stress. The confidence interval of change of 90 s^{-1} and 240 s^{-1} blood viscosity, as well as plasma viscosity, was narrow, that could be explained by intrinsic inaccuracy of the instrument (3% by the manufacturer). When stored at 4ºC, 90 s^{-1} and 240 s^{-1} blood viscosity raised significantly even after 24 hours, while this tendency was visible only after 48 hours for 10 s^{-1} blood viscosity – probably due to the higher standard deviation. Plasma viscosity was clearly elevated after 24 hours of storage at 4ºC as well. Contrarily, Casson viscosity seemed to be stable on average, as well as yield shear stress, however, the confidence intervals of change were very broad.

Whole blood viscosity is determined by four main factors: plasma viscosity, hematocrit, erythrocyte deformability and aggregation. Capillary hematocrit did not change significantly throughout the experiment. Plasma viscosity is mainly determined by soluble macromolecules, most importantly fibrinogen. By the degradation of these molecules one could expect the decrease of plasma viscosity and not the increase as demonstrated. On the other hand, degradation may mean changing from a compact conformation into a more extended conformation that can increase viscosity. Since plasma was stored together with cells, erythrocytes may have discharged surface attached or intracellular molecules into plasma increasing its viscosity. At low shear rates erythrocyte aggregation is considerable and responsible for a great deal of whole blood viscosity. The increased low shear viscosity suggests increased aggregation, which contradicts our results with aggregometry but concordant with the results of other authors. Viscosity values at 90 s^{-1} are interpolated in our instrument, while 10 s^{-1} and 240 s^{-1} values are extrapolated, and less accurate. Casson viscosity and yield shear stress are derived parameters obtained by curve fitting. Not surprisingly, these results showed a broader confidence interval.

Conclusions

Both blood and plasma viscosities remain unchanged during 6 hours of storage at room temperature, whereas overnight storage at 4ºC should be avoided. Erythrocyte deformability does not change significantly during 2 hours of storage at room temperature or overnight storage at 4ºC. Longer storage however may influence it. General centrifugation has no effect on deformability, while washing with buffer significantly decreases it. Erythrocyte aggregation index and threshold shear rate with LORCA is stable during 6 hours of storage at room temperature, but they are unreliable after overnight storage. Myrenne M and M1 indices change even after two hours of storage at room temperature.

5. Methodological problems with ektacytometry: data reduction

Introduction and methods

Deformability allows erythrocytes to pass through capillaries of the microcirculation with a diameter smaller than the cells themselves. Ektacytometry is a method where cells are subjected to controlled shear stresses, and their shape change is quantified. We use a LORCA device in our laboratory. The device consists of a standing bob and a rotating cup that builds up Couette-flow, a nearly homogenous shearing field. Erythrocytes are suspended to a low hematocrit in a known viscosity medium (25 µl blood/5 ml medium, 0.2% Hct). When erythrocytes are subject to a homogenous shear flow and the suspending phase viscosity exceeds intracellular viscosity, erythrocytes deform into an ellipsoidal shape and the membrane performs tank-treading motion around the cytoplasm. The long axis is tilted slightly from the axis of vorticity. Deformed cells pass in front of a laser beam causing diffraction. Deformation is quantified by the elongation index (EI) that is calculated by the following formula where A and B represent the major and minor axes of the obtained elliptical diffraction pattern: EI=(A-B)/(A+B). Changing the revolution of cup, EI can be determined at a wide range of shear stresses (SS). The set of SS-EI datapoints is called ektacytogram.

Raw measurement results are hard to handle, interpret and compare. A deterministic relation can be seen between EI and SS. Data reduction methods that describe this relationship with few parameters and without loss of information, would be welcome. We reviewed two such methods in the literature.

EI was measured at shear stresses between 0.30 to 30 Pa in 100 logarithmic steps to obtain a high-resolution ektacytogram. The ektacytogram was considered representative for healthy volunteers. Regression curves were fit on the dataset using Wolfram Mathematica 6.0 package. The algorithm returned parameter values, where the sum of square of residuals between original datapoints and regression curve was minimal. Goodness of fit was represented by the r2 value, which is the proportion of the total variance of raw data explained by the regression curve. To assess the systematic deviation we examined the distribution of residuals according to the shear stress.

The Lineweaver-Burke method

When both the SS and EI values are plotted on a linear scale, the concave ektacytogram is similar to the plot of first order enzyme kinetics that can be solved by the Lineweaver-Burke reciprocal transformation. In this method 1/EI is plotted against 1/SS then the relationship is described with linear regression. EI_{max} is the deformation value at a theoretically infinite shear stress and $SS_{½}$ is the shear stress that causes half-maximal deformation ($EI_{max}/2$) (Figure 1/a).

$$\frac{1}{EI} = \frac{SS_{1/2}}{EI_{max}} \frac{1}{SS} + \frac{1}{EI_{max}} \qquad EI = EI_{max} \frac{SS}{SS + SS_{1/2}}$$

The least squares method used for regression minimizes the absolute difference between the measurement points and the fitted line. The same absolute difference that causes merely a small relative difference at high 1/SS and high 1/EI causes a huge relative difference at low 1/SS and low 1/EI. The regression analysis, however, is insensitive for this phenomenon. Importance of low (1/SS; 1/EI) datapoints is underweighted. The closer the EI to zero at low shear stress is, the higher the 1/EI is, which 'pulls away' the regression line and, as a consequence, EI_{max} is often significantly overestimated. Occasionally, it may even exceed 1, the theoretical maximum of EI (Figure 1/b-c). A significantly better fit may be achieved if points with high 1/EI (i.e., points below 1 Pa shear stress) are omitted from the analysis. However, it would mean a significant loss of information, besides, these points cannot be reproduced using the formula.

The method cannot be used if 0 or negative EI values are present (EI can be negative due to the residual orientation phenomenon, most commonly an artifact).

Technically, it is also possible to fit the non-reciprocal curve (see above) directly on the SS-EI plot. This method respects high (SS; EI) points more and thus EI_{max} is more reliable. However, this way the importance of low (SS; EI) points is underweighted. Clustered residuals between datapoints and the non-reciprocal curve (Figure 1/d) denote to systematic deviation. At low shear stress values EI is overestimated. Numbers in the figure are absolute values; in case EI is small, the relative difference is even more pronounced. At high shear stresses, residuals tend to negative values, EI_{max} is thus underestimated. The function cannot properly handle zero or negative values either. The non-reciprocal curve fitting technique provides a better estimation of EI_{max} than the original Lineweaver-Burke method. However, at low shear stresses, it is not provide a better fit. In addition, non-linear curve fitting may be a more difficult task than a simple reciprocal transformation and linear regression.

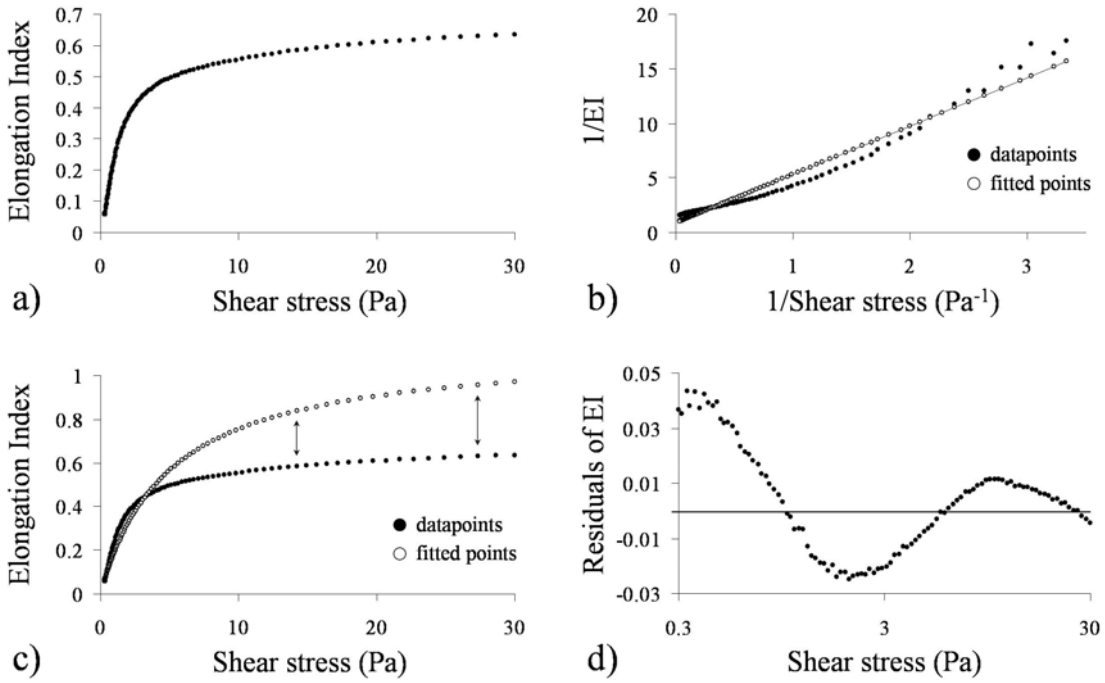

Figure 1: Data reduction according to Lineweaver-Burke transformation. **a)** Linear-linear scaling concave ektacytogram. **b)** Reciprocal transformation and fitted regression line. EI_{max}=1.133, $SS_{½}$=5.029 Pa. **c)** Values measured and calculated according to Lineweaver-Burke transformation. EI_{max} is overestimated. **d)** Residuals between the datapoints and the non-reciprocal regression curve (EI_{curve}-EI_{data}).

The Streekstra-Bronkhorst method

When SS is logarithmically and EI is linearly scaled, the ektacytogram is S shaped (Figure 2/a). Streekstra and Bronkhorst aimed to describe it with an $x^m/(x^m+1)$ type function. This basic function is appended with constants that expand and shift the curve along the axes to position for a good fit.

$$EI = (EI_{max} - EI_{min}) \frac{\left(\frac{SS}{SS_{1/2}}\right)^m}{\left(\frac{SS}{SS_{1/2}}\right)^m + 1} + EI_{min}$$

EI_{max} and EI_{min} are the upper and lower asymptotes of the curve. EI_{max} represents deformation at infinite shear stress, while EI_{min} is the EI at 0 shear stress. $SS_{½}$ is the shear stress needed to obtain mean deformation, $(EI_{max}+EI_{min})/2$. Parameter m is in connection with the steepness of the curve. Streekstra-Bronkhorst method can handle negative EI values as well.

A theoretical problem is present in this approach. Since the *log*SS–EI function is S-shaped here, the formula should hold *log*SS and *log*$SS_{½}$, instead of SS and $SS_{½}$. The present formula approximates the linear-linear scaling plot, which is not S-shaped but concave. It is an irony of mathematics, that even this way the obtained fit is very strong. $x^m/(x^m+1)$ is a

flexible function, that is concave if m≤1, and turns into S-shaped only if m>1 (Figure 2/b). The m values of fitted curves were below 1, proving that they actually fit on the concave linear-linear scaling ektacytogram (Figure 2/c). Parameter m thus has no picturesque meaning, it is only a necessary parameter for the forced fitting.

Residuals show systematic deviation between the datapoints and the regression curve: both EI_{max} and EI_{min} are underestimated (Figure 2/d).

We modified the Streekstra-Bronkhorst formula by adding logarithms, but fitting of this formula shows other problems (the expression is undefined for shear stresses below 1 Pa, this can be overcome by temporary rescaling of SS). The modified method gives a similar fit as the original Streekstra-Bronkhorst formula; however, it results in similar systematic deviations as well. Our method is not more useful, than the original one; on the other hand, it is more complicated. Therefore, we suggest the use of the Streekstra-Bronkhorst method for data reduction further on.

The data reduction parameters are to describe deformation at any shear stress, thus they theoretically should not depend on the shear stress range of the measurement. To check this, we discarded the upper or lower shear stress region of the dataset, and performed the curve fitting on the truncated subsets. Parameters changed considerably. To correctly assess parameters special parts of the curve are needed.

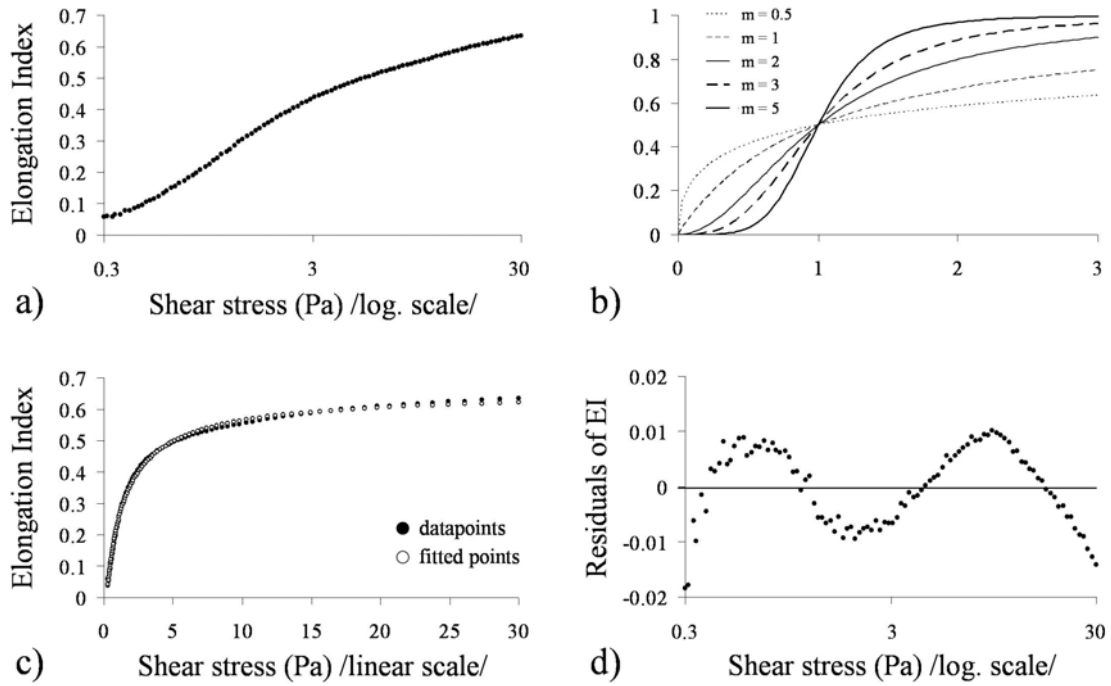

Figure 2: Data reduction according to the Streekstra-Bronkhorst method. **a)** Log-linear scaled S-shaped ektacytogram. **b)** The flexible $x^m/(x^m+1)$ function. Concave if m≤1, S-shaped if m>1. **c)** Datapoints and the fitted curve. EI_{max}=0.660, EI_{min}=-0.161, $SS_{½}$=1.061 Pa, m=0.899. **d)** Residuals between the datapoints and the regression curve (EI_{curve}-EI_{data}).

General comments

r^2 represents the strength of the fit, which was extraordinarily high for both methods. It is possible because the ektacytogram is a precise, noise-free curve where the stochastic deviation from the supposed deterministic baseline is minimal. This makes detection of small systematic deviations possible. High r^2 value guarantees that the fitted curve closely describes the original datapoints and that any of the measured points can be re-calculated using the formula with high accuracy. As neither the regression curve nor the ektacytogram have sudden outliers, any other random points within the measurement range can be calculated accurately. However, the high r^2 value does not mean that the methodology can accurately predict datapoints that fall outside of the measurement range, such as maximal erythrocyte deformation. Dependence of parameters on the measured SS range refers to this as well. The precision of estimation may improve by having more EI parameters available, i.e., by extending the measurement range. At the lower end it is limited by the increasing noise/signal ratio; when applying extremely small shear stresses, the Brownian movement of cells may be a considerable confounding factor. Extreme high shear stresses, on the other hand, will cause irreversible damage to erythrocytes and thus the EI values will be unreliable and irrelevant.

The least squares method considers the absolute magnitude of residuals. Difference between 0.08 and 0.10 has the same importance as that between 0.58 and 0.60. However, while the former represents a 20% relative difference, the latter only stands for a 3.3% difference. Expressions like relative difference, relative error or standardized difference are commonly used. It is to be argued, however, if they have any meaning when considering EI. These expressions are valid for ratio scale variables. Elongation index is rather an interval scale variable limited at both ends, where the same difference between values does not necessarily represent the same magnitude of biological change. EI can be easily transformed into the length/width ratio of the ellipse: L/W=(1+EI)/(1-EI). The increase of EI from zero to 0.3 means deformation from circle to an ellipse with a diameter ratio of 1.86. Increase of EI from 0.8 to 0.9 means deformation from diameter ratio of 9 to 19. It is also meaningless to calculate the ratio of a positive and negative EI.

Conclusions

The Lineweaver-Burke transformation overestimates maximal erythrocyte deformation if EI values measured below 1 Pa are included. A non-reciprocal version of the formula estimates EI_{max} more accurately, but underweights the importance of data at low shear stresses. The Streekstra-Bronkhorst method provides a more efficient data reduction. Though the theoretical built of the formula is mistaken, its practical use is not to be questioned. Information can easily be reproduced using only a few parameters that have expressive meaning. However, it should be remembered that calculated parameters might be inaccurate, and both the maximal and minimal deformation values are slightly underestimated.

6. Methodological problems with ektacytometry: suspending medium viscosity

Introduction

Ektacytometry is a method to describe erythrocyte deformation. Elongation index quantifies *deformation*. The expression *deformability* is used for measurements among standardized circumstances (temperature, suspending medium viscosity, osmolarity, pH etc.). Deformation of cells of the same sample, and thus with same deformability, can be different among changed conditions.

We use polyvinylpyrrolidone (PVP) powder solved in phosphate buffered saline as suspending medium. Preparation of the solution and setting of viscosity is difficult, thus standard environment is hard to achieve. As authors in the literature use various viscosity media, comparison of deformation results has limitations. We studied how the change in medium viscosity influences deformation.

The device determines revolution of cup according to the medium viscosity. Improper setting of medium viscosity leads to false shear stresses and elongation index values. We seeked a method to correct such results retrospectively.

Samples and methods

We used heparin anticoagulated blood samples of 11 healthy volunteers. Five batches of PVP solutions were prepared, their viscosities were measured by Oswald viscometer to be 13.5, 17.5, 20.9, 25.1 and 37.8 mPas, respectively. Measurements were performed within 2 hours after blood sampling. EI was measured at shear stresses ranging from 0.30 to 30 Pa in 25 logarithmic steps.

Each blood sample was suspended in each medium. Each mixture was measured 3 times sequentially without being replaced between measurements. Once the viscosity value was configured correctly, the other two times false – preferably one lower and one higher – values were given, creating controlled errors.

LORCA sets the shear rate of Couette-flow at $\gamma=SS_{nominal}/\eta_{nominal}$. This results in shear stress $SS_{real}=\gamma*\eta_{real} = SS_{nominal}*\eta_{real}/\eta_{nominal}$. If viscosity is given correctly, then $\eta_{real}=\eta_{nominal}$, thus $SS_{real}=SS_{nominal}$. Contrary, if incorrect viscosity value is given, measurement is performed on shear stresses different from the nominal shear stresses resulting in false ektacytogram. First, false ($SS_{nominal}$; EI) datapoints were transformed to correct (SS_{real}; EI) datapoints. Then, the transformed datapoints were interpolated to common SS values, so that plots could be compared. Datapoints for smallest or largest shear stresses could not be obtained by interpolation, only by less accurate extrapolation, therefore they were omitted. Agreement of correctly measured and transformed, incorrectly measured points was tested by Bland-Altman analysis on each shear stress.

Ektacytograms of the same samples measured in the five media were plotted together. The influence of medium viscosity on ektacytograms was described semiquantitatively, and the changes of Streekstra-Bronkhorst parameters were calculated as well.

Results and discussion

Correctly measured and transformed, incorrectly measured datapoints showed an excellent agreement, no systematic bias was present and the stochastic error was within the working precision of LORCA as well. As the measurements were performed with the same viscosity medium, physical conditions and thus EI values were identical at the same real shear

stresses. The only different thing was the nominal value of SS. It is not surprising that this strictly mathematical error could be reverted. However, there are limitations for the method. We measured 25 datapoints each case to obtain a high resolution, thus interpolation was accurate. If one has less points (the default number in LORCA is 9), this may be inaccurate. Moreover, at least one side point cannot be interpolated, hence it is lost.

We also tried to transform Streekstra-Bronkhorst data reduction parameters of the incorrect measurements. EI_{max} and EI_{min} were left unchanged, as maximal and minimal deformation ought to stay unchanged among same physical conditions. $SS_{½}$ was transformed to $SS_{½}*\eta_{real}/\eta_{nominal}$ for the reason previously discussed. Parameter m was left unchanged as well. The transformed parameters were biased from correctly measured parameters according to Bland-Altman analysis. If nominal viscosity was higher than real, EI_{max} became underestimated, EI_{min}, $SS_{½}$ and m became overestimated. If nominal viscosity was lower than real, parameters were biased in the opposite direction. We rather suggest correcting the original data, and not the derived parameters, whenever possible.

In our study, if samples were measured in higher viscosity media, ektacytograms were generally shifted toward higher EI values. Streekstra-Bronkhorst EI_{max} and m parameters showed significant positive, while $SS_{½}$ showed negative correlation with viscosity. EI_{min} also showed an increasing tendency toward higher viscosity values. We tried to create a transformation rule for both raw EI values and Streekstra-Bronkhorst parameters, so that we could calculate the values at an arbitrary viscosity from the values at a known viscosity. Describing the relationship between viscosity and EI values with a 1/x type hyperbole and using a set of constants seemed moderately successful if the average values of all samples were considered. This rule, however failed to work in individual cases, and recalculated constants behaved so differently that the model had to be discarded.

Ektacytograms of various samples behaved differently in various viscosity media. In some samples ektacytograms tend to diverge at high shear stresses; in others, however, this is not so marked. Similarly, convergence of curves at low shear stresses is present in a different degree. In most samples curves run parallel, sometimes, however, they can cross each other. This highlights the importance of standardizing measurements. Authors use a wide range of viscosities, and their results will not be easy to compare. On the other hand, the diversity as samples respond to medium viscosity may also open a new scope for ektacytometry. The workgroups at Peking University managed to apply low viscosity ektacytometry to elicit membrane fluidity properties. High viscosity ektacytometry may give information about the stress-tolerance of erythrocytes, but transition zone near the inner viscosity supposedly possess useful information as well. And as osmotic gradient ektacytometry revealed much about erythrocyte properties, so may do a possible future method “viscosity gradient ektacytometry”.

NOVEL FINDINGS

1. Hematocrit/blood viscosity ratio may help predicting cardiac morbidity and mortality.
2. The introduced method describes hematokrit-blood viscosity profile using small sample volume in quick and simple way, and it helps to determine virtual optimal hematocrit.
3. Our results suggest that virtual optimal hematocrit may help to assess the global optimal hematocrit of patients, if information about hemodynamics can be obtained.
4. We demonstrated how long samples may be stored before measurement without the bias of results:
    - Whole blood viscosity and plasma viscosity are stable for 6 hours at room temperature. Avoid 24 hours of storage at 4°C!
    - Erythrocyte aggregation parameters are stable for 6 hours in LORCA, and for less than 2 hours in Myrenne at room temperature. Avoid 24 hours of storage at 4°C!
    - Erythrocyte deformability is stable for 2 hours at room temperature or for 24 hours at 4°C
5. We demonstrated some limitations of data reduction methods for ektacytometry:
    - In case of the original Lineweaver-Burke transformation, do not use shear stresses below 1 Pa! The non-reciprocal method is inaccurate for these points as well.
    - The Streekstra-Bronkhorst method produces systematic deviations as well. Maximal and minimal deformation is slightly underestimated.
6. We introduced a method to correct errors made by incorrectly giving medium viscosity.
7. We demonstrated that the relationship between deformability and medium viscosity varies individually, thus investigating this relationship may provide further information.

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ABBREVIATIONS

PV	Plasma viscosity
BV	Whole blood viscosity
Hct	Hematocrit
$Hct_{opt,v}$	Virtual optimal hematocrit
$Hct_{opt,g}$	Global optimal hematocrit
Hct/BV	Hematocrit/blood viscosity ratio
EI	Elongation index
SS	Shear stress
PVP	Polyvinylpyrrolidone
AI	Aggregation index
η	Viscosity
γ	Shear rate

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