In vitro hemorheological studies focusing on erythrocyte deformability and aggregation

Ph.D. dissertation

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II. List of abbreviations

AFRW	alcohol-free red wine
AI	aggregation index, parameter of LORCA aggregometer
CVD	cardiovascular disease
EI	elongation index, parameter of LORCA ektacytometer
EI_{max}	maximum of the elongation index at infinite shear stress
HbSS	hemoglobin S
ISC	irreversibly sickled cell
LDL	low-density lipoprotein
LORCA	Laser-assisted Optical Rotational Cell Analyzer
M	erythrocyte aggregation at stasis, parameter of Myrenne
M1	erythrocyte aggregation at very low shear, parameter of Myrenne
NO	nitric-oxide
PBS	phosphate buffered saline
PMS	phenazine methosulfate
PS	physiological saline
RBC	red blood cell
RSC	reversibly sickled cell
RW	red wine
SCD	sickle cell disease
SS	shear stress
$SS_{1/2}$	shear stress required for the one-half of the maximal elongation
γ	threshold shear rate, parameter of LORCA aggregometer

III. Prologue

1. The cardiovascular risk and the role of hemorheology

The Framingham Study and other epidemiological investigations have revealed numerous cardiovascular risk factors (e.g., genetic background, male gender, age, hypertension, diabetes mellitus or impaired glucose tolerance, hyperlipidemia, obesity, smoking and the lack of physical exercise). In contrast to the above mentioned "classic" factors, several studies have reported the importance of cardiovascular prevention with the basic principles of risk reduction (e.g., adequate lifestyle, risk screening, combined medicinal and operative therapies). Those examinations have described that avoidance of tobacco and of overweight, regular physical activity and healthy food consumption including Mediterranean diet with moderate red wine intake are primarily needed for the risk-free lifestyle of cardiovascular health [1]. In spite of these fundamental observations, several factors have been remained in the pathomechanism of cardiovascular diseases which requires deeper investigations, such as the properties of circulating blood.

Blood is a non-Newtonian suspension containing cells (e.g., erythrocytes, leukocytes and platelets), lipid components, proteins, carbohydrates and electrolytes. Blood flow is characterized by several hemorheological parameters, such as hematocrit (i.e., volume fraction of blood cells), plasma and whole blood viscosity (i.e., intrinsic resistance to flow generated by internal friction between nearby fluid layers), plasma proteins (e.g., fibrinogen and some globulins), erythrocyte deformability (i.e., the ability of red blood cells to deform in response to mechanical forces) and aggregation (i.e., rouleaux formation under low flow conditions).

Numerous investigations have presented that altered hemorheological parameters can impair microcirculation leading to development of various diseases [2, 3]. Furthermore, several literature reports have clearly shown that hemorheological parameters (e.g., hematocrit, plasma fibrinogen and blood viscosity) can also be considered as potential risk factors, and abnormalities of these parameters contribute to the development of cardiovascular diseases [4-6].

Other hemorheological parameters, such as red blood cell deformability and aggregation have also been under both basic science and clinical interest; reduced deformability and increased aggregation can affect the rheological behavior of blood and can impair *in vivo* tissue perfusion.

2. Erythrocyte deformability

The form of a normal human red blood cell is a biconcave disc ~8 μm in diameter, ~2 μm thick at the rim, and ~1 μm thick at its center [7]. These cells are extremely deformable as they progressively elongate under shear stress (i.e., erythrocyte deformability is a major determinant of high shear viscosity of blood) and form ellipsoids with their long axis aligned with the flow. Thus erythrocytes are capable of entering and transiting blood capillaries with diameters as small as ~4 μm. When the cells deform their surface area remains fixed; the red blood cell membrane resists area dilation and ruptures above 2-3% area expansion [8].

There is a general agreement regarding the factors affecting erythrocyte deformability: cell shape and membrane surface area to volume ratio as "extrinsic" factors; membrane viscoelastic properties and cytosolic viscosity as "intrinsic" factors [9-11]. Deviations from the normal resting biconcave shape, decreased area to volume ratio, higher membrane shear modulus and viscosity or elevated cytoplasmic viscosity tend to reduce deformability [2].

Erythrocyte deformability can be modified by several structural and functional alterations of erythrocytes generated by genetic or environmental factors. Changes in strictly regulated properties of blood (e.g., osmotic pressure, pH, etc), mechanical and oxidative damages induced by various internal or external sources (e.g., ischemia/reperfusion injury, mitochondrial leakage, activated leukocytes, iron overload caused by transfusions, etc), parasite infection (i.e., malaria caused by the genus Plasmodium) and genetic disorders (hemoglobinopathies, genetic modifications of red blood cell membrane proteins, enzyme deficiencies of erythrocyte metabolism, etc) are associated with decreased red blood cell deformability [2].

Abnormal red blood cell deformability is especially notable in sickle cell disease in which the erythrocytes become rigid at reduced oxygen levels due to intracellular polymerization of sickle hemoglobin leading to occlusions in microvessels and impaired tissue perfusion resulting in painful crisis, infarctions of various organs and increased resistance to flow in the lungs [12].

3. Erythrocyte aggregation

Erythrocyte aggregation is a major determinant of *in vitro* hemorheology occurring in either plasma or solutions with large polymers (e.g., dextran $\geq$ 40 kDa). During the process, red blood cells reversibly form linear (i.e., like stack of coins termed rouleaux formation) or branched aggregates. Under *in vivo* circumstances erythrocyte aggregation occurs at low shear forces thus determining especially the low shear viscosity of blood [13].

Erythrocyte aggregation is characterized by red blood cell aggregability (i.e., the intrinsic cell characteristics) and the concentration of macromolecules, such as proteins in plasma (e.g., fibrinogen and some globulins) or neutral polymers in suspensions (e.g., dextran) [14-17].

At these days, two parallel models explain the process of aggregation. The bridging theory claims that erythrocyte aggregation occurs when disaggregating forces are not capable to interfere the adsorption of macromolecules to the nearby cell surfaces, while the depletion model suggests that the decreasing protein or polymer concentration creates an osmotic gradient between two adjacent erythrocytes leading to depletion interaction [18-20].

Although increased erythrocyte aggregation has been observed in various clinical diseases (e.g., hypertension, diabetes mellitus) [21, 22], all mechanisms of the process and the relations between different pathological states and red blood cell aggregation have not been completely understood.

4. Aim of the studies

These studies were designed to investigate the possible alterations of erythrocyte deformability and aggregation in two *in vitro* experiments: 1) effects of red wine, alcohol-free red wine extract and ethanol was examined; 2) light scattering results of red blood cells in ektacytometry were also analyzed.

IV. Methodology

1. Early observations in hemorheology

First hemorheological observations were occurred in 1674 and performed by Anthony van Leeuwenhoek (Delft, the Netherlands), the inventor of microscope, who discovered the fact that erythrocytes have to deform for traversing capillaries [23] and also realized that red blood cells tend to form aggregates under low flow conditions [24]. In fact, the scientific interest appeared and turned to hemorheology just after the report describing the thesis of blood viscosity of Fåhraeus and Lindqvist in 1931 [25].

Until these days, many investigations have been performed and numerous instruments have been manufactured for better understanding the structural and functional integrity of cellular and soluble components of blood as well as the different blood flow properties.

2. Erythrocyte deformability measurements

Various approaches to the measurement of red blood cell deformability have been developed, such as filtration models requiring the cell to enter and pass through a small orifice, micropipette techniques describing deformability during a complete erythrocyte aspiration into a narrow glass tube, or those techniques (i.e., termed laser diffraction ellipsometry, also known ektacytometry) where cells are exposed to fluid shear stresses in defined flow fields [26-30].

In these experiments, erythrocyte deformation in response to shear forces was determined by a LORCA ektacytometer (Laser-assisted Optical Rotational Cell Analyzer; R&R Mechatronics, Hoorn, Netherlands). In this instrument a dilute suspension of erythrocytes (~ 2 x 10^7 cells/ml) in a viscous medium is placed in the gap of a Couette shearing system (i.e., created by an outer, rotating cylinder and an inner, static cylinder) having a laser-diode projected through the gap.

The presence of red blood cells in the gap diffracts the laser light that creates a diffraction pattern on a diaphragm changing from circular to elliptical as cells deform and elongate. The pattern is captured and analyzed by a video camera and a computer system that calculates an elongation index (EI) as the (length - width) / (length + width) of the pattern for each shear stress (SS). Results are shown on an elongation index-shear stress (EI-SS) diagram [31] (Fig. 1A).

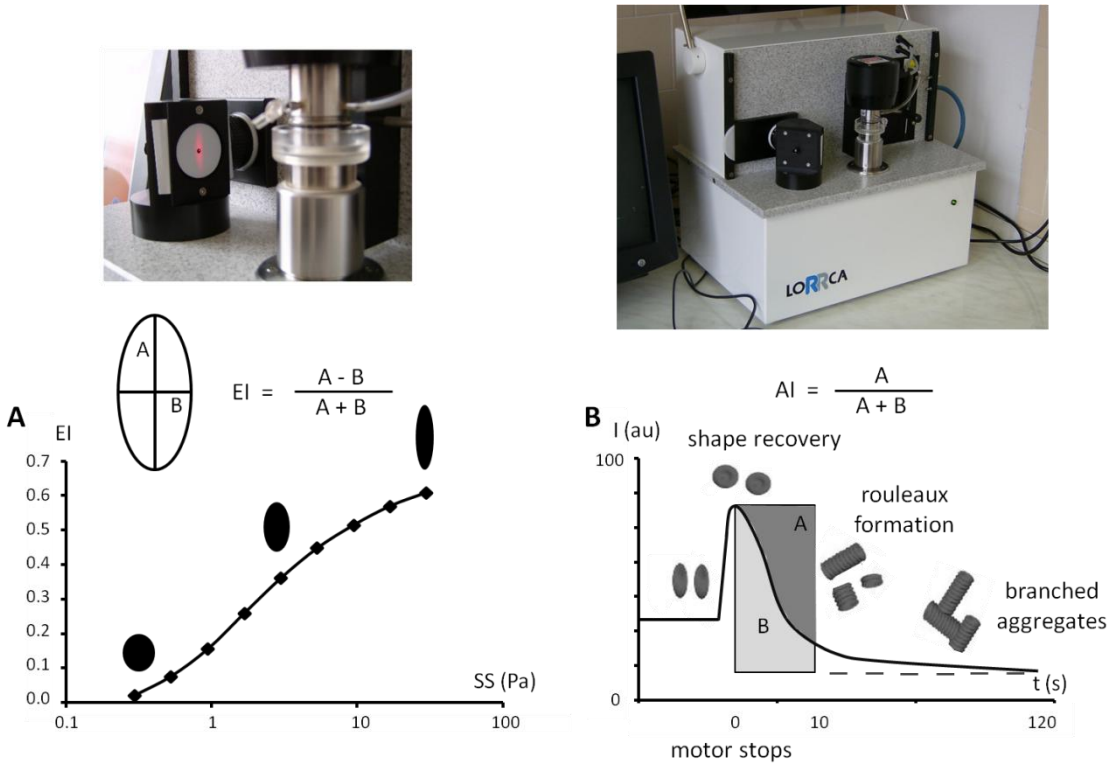

Fig. 1: *The LORCA (Laser-assisted Optical Rotational Cell Analyzer) machine and its two cylinders with the laser beam creating an ellipsoidal diffraction pattern on a diaphragm.* ***A)*** *Ektacytometric investigation of erythrocyte deformability with an elongation index-shear stress (EI-SS) diagram and the analysis of the diffraction pattern.* ***B)*** *Illustration of laser backscattering intensity vs. time curve (syllectogram) and the process of erythrocyte aggregation with the analysis of the diagram. This figure is partly based on the illustrations of the Handbook of Hemorheology and Hemodynamics, IOS Press, Amsterdam, pp. 250 and 256, 2007.*

3. Erythrocyte aggregation measurements

In this study, red blood cell aggregation was measured with two instruments employing different approaches of the method of syllectometry (i.e., measuring light intensity changes caused by the process of erythrocyte aggregation) [32]: 1) LORCA aggregometer operating with laser backscattering; 2) Myrenne aggregometer using infrared light transmission [30].

Red blood cell aggregation can be determined with a LORCA aggregometer detecting the laser backscattering from the aggregating blood. Erythrocytes are placed in the gap of the instrument and disaggregated at a high shear rate (500 s^{-1}) which reduces rapidly to zero. Backscattering of laser light suddenly increases (i.e., sheared and elongated red blood cells recover their normal biconcave shape) then decreases during the process of aggregation (i.e., larger aggregates reflect less light than single cells) that is characterized by the aggregation index (AI) calculated from the areas A and B of the diagram (AI=A/A+B) during the first 10 seconds of the measurement [33-35] (Fig. 1B). Another sensitive parameter of red blood cell aggregation is called threshold shear rate (γ) describing the smallest shear rate which is required for the complete disaggregation of erythrocytes [33].

Erythrocyte aggregation can also be measured with a Myrenne aggregometer (model MA-1, Myrenne GmbH, Roetgen, Germany) that employs and measures the infrared light transmission through an erythrocyte suspension between a transparent plate and a cone. Cells are initially disaggregated by the cone at high shear (600 s^{-1}) following which shear is abruptly stopped or reduced to 3 s^{-1} and light transmission integrated for 10 seconds. The instrument provides two dimensionless indices of red blood cell aggregation (M, aggregation at stasis; M1, at very low shear); both M and M1 increase with enhanced aggregation (i.e., quicker process or larger aggregates let more light to pass through the sample) [33, 36] (Fig. 2).

Fig. 2: *A Myrenne aggregometer MA-1 demonstrating the experimental chamber with the transparent plate-cone shearing system.*

V. *In vitro* hemorheological effect of red wine, alcohol-free red wine and ethanol

1. Introduction

1.1. The "French Paradox"

Cardiovascular diseases (CVD) are among the most frequent causes of morbidity and mortality in the developed countries. Until these days, numerous risk factors (listed on page 4) have been determined in the pathogenesis of CVD [37-39].

Several epidemiological studies have revealed that total mortality is not but CVD-related death is substantially lower in France than in other industrialized Western-European countries, although consumption of saturated fats and level of blood cholesterol are higher, while other major risk factors, such as smoking and hypertension are similarly prevalent in France as in other developed regions [40, 41].

In contrast to the harmful pathological consequences of chronic drinking of large amounts of alcoholic beverages, further epidemiological studies have demonstrated a J-shape relationship between CVD mortality and consumed alcohol amount [42-45] and shown that regular but moderate (i.e., not more than 10-20 g alcohol per day) red wine (RW) consumption results in a decreased risk of coronary heart disease [46, 47], heart failure [48], intermittent claudication [49] and stroke [50]. On the other hand, binge- or heavy alcohol drinking leads to higher mortality risk in CVD [51-53].

According to other studies, wine consumption is associated with higher beneficial cardiovascular effects compared to other forms of alcohol; low to moderate intake of wine decreases the mortality risk of patients suffering from CVD, while similar amount of spirits drinking increases, but beer consumption does not show any influence on it [54, 55]. Furthermore, this beneficial protective effect depends on the type of wine; mortality rates in the RW drinking Mediterranean regions is lower than in Alsace, a white wine drinking area of France [56].

This chapter is based on the following scientific articles:

- Rábai M, Tóth A, Kenyeres P, Márk L, Márton Zs, Juricskay I, Sümegi B, Tóth K. Vörösbor és alkoholmentes vörösborkivonat kedvező in vitro haemorheológiai hatásai. *Érbetegségek* **2**, 45-52, 2009.
- Rabai M, Toth A, Kenyeres P, Mark L, Marton Zs, Juricskay I, Toth K, Czopf L. In vitro hemorheological effects of red wine and alcohol-free red wine extract. *Clin Hemorheol Microcirc* **44**, 227-236, 2010
- Rabai M, Detterich JA, Wenby RB, Toth K, Meiselman HJ. Effects of ethanol on red blood cell rheological behavior. *Clin Hemorheol Microcirc*, accepted for publication.

This phenomenon (i.e., beneficial cardiovascular effects of regular and moderate RW consumption) has been termed the “French Paradox” [41].

1.2. Components of red wine

It is assumed that the favorable cardiovascular effects of RW originate in its non-alcoholic (phenolic) and alcoholic (ethanol) components [57].

Non-alcoholic component of RW contains anthocyanins and polyphenols. Anthocyanins, such as delphidin and malvidin are responsible for the color of wines, while polyphenols are believed the main source of the cardiovascular protection.

The most potent polyphenols of RW, such as resveratrol, katechin and quercetin have been extensively studied. Several researchers have demonstrated that the amount of polyphenols, especially the resveratrol content, depends on vintage year and variety [58, 59], while other authors have shown that winemaking technology and winery region are determinant factors as well [60-62]. Thus different RW may have different influence on health, as resveratrol presumably plays a role in the CVD mortality risk reduction.

Favorable cardiovascular effects of polyphenols have been widely investigated and their antioxidant properties are well-known [63]. In addition to the decreased oxidation of low-density lipoproteins (LDL) [64] and expression of LDL receptors [65], polyphenols induce the nitric-oxide (NO) production and reduce the platelet aggregation plus the production of proinflammatory eicosanoids [66, 67].

Alcohol-free red wine (AFRW), an extract of RW containing phenolic components without ethanol, has been particularly used in animal model investigations. An ischemia-reperfusion rat model measurement has proven that AFRW treatment improves the ventricular functions and reduces the area of postinfarction remodeling [68]. Some other rat experiments have reported that AFRW feeding decreases the thrombotic tendency [69] and the degree of oxidative stress [70], while a human study has shown that AFRW ingestion inhibits the oxidation of LDL [71].

Several studies of RW consumption have confirmed that, in addition to the antioxidant phenolic components, ethanol plays a role in the beneficial cardiovascular effects of moderate red wine intake as well. Ethanol favorably modifies hemostasis leading to reduced levels of certain coagulation factors (e.g., fibrinogen, factor VII and von Willebrand factor) and of platelet function [72, 73]; enhanced fibrinolysis due to elevated levels of tissue-type plasminogen activator has also been demonstrated [73, 74]. Alterations of plasma lipid profiles with an increase in high-density lipoprotein and a decrease in LDL cholesterol concentrations have been reported [75, 76]. Furthermore, ethanol also enhances the production of the vasodilator endothelial NO [77].

1.3. Effect of red wine and its components in hemorheology

Several studies have demonstrated the above mentioned inverse association between RW or alcohol intake and cardiovascular events, but the source of this cardioprotective effect is still not completely known.

Only a small number of *in vivo* and *in vitro* experiments have been performed to evaluate the effects of RW, polyphenols and ethanol on different hemorheological factors. Unfortunately the results are not in complete agreement. While *in vitro* studies exploring the effects of ethanol addition to blood indicate no changes of hematocrit or whole blood viscosity [78-80], alcohol consumption leads to dehydration without increased hematocrit [79, 80] but with an elevation of whole blood and plasma viscosity [78, 81-83]. On the other hand, several studies have demonstrated that regular but modest alcohol ingestion is associated with a decreased level of plasma fibrinogen [73, 84] which was correlated with a reduction of plasma viscosity [85]. In contrast, a recent study has reported no changes of fibrinogen, hematocrit or blood viscosity after moderate RW and vodka consumption for two weeks [86].

Given the current uncertainty and the lack of data regarding the specific hemorheological consequences, the present *in vitro* study was designed to further explore possible effects of red wine and its major components on red blood cell (RBC) deformability and aggregation.

2. Methods

2.1. Red wine, alcohol-free red wine extract and ethanol

Throughout the measurements, a 2002 Merlot (Polgar Winery, Villany, Hungary) red wine was applied since previous chromatographic measurements have proved its high resveratrol content (approximately 14 mg/l), while the alcohol concentration was around 13% [62, 87]. For alcohol-free red wine extract measurements wine sample was vacuum distilled until the disappearance of alcohol. The extract was rediluted with distilled water, thus its osmolality became physiological plus the polyphenol and anthocyanin content remained similar to the original red wine [68]. Alcohol experiments were performed with reagent grade ethanol (Sigma-Aldrich Co., St. Louis, MO, USA), while physiological saline (PS) and isotonic phosphate buffered saline (PBS, 290 mOsm/kg, pH = 7.4) were utilized as a diluent control.

2.2. Blood samples

Venous blood samples were obtained by sterile venipuncture with a 21-gauge butterfly infusion set using a minimal tourniquet from 13 healthy volunteers into Vacutainer tubes coated with lithium heparin for the red wine and polyphenol measurements. For the ethanol experiment blood was taken from 7 adult laboratory personnel and anticoagulated with ethylenediamine-tetraacetic acid (1.5 mg/ml). Blood donors did not consume any ethanol-containing products within 24 hours of sampling. Red wine study was supported by the Regional Ethics Committee, University of Pecs, Pecs, Hungary, while the experiment with ethanol was approved by the Human Subjects Institutional Review Board, University of Southern California, Los Angeles, CA, USA.

2.3. Erythrocyte deformability and aggregation testing

Following the RBC sample preparation (see below), erythrocyte deformability was studied with a LORCA ektacytometer (see page 8), while aggregation was measured employing a Myrenne and a LORCA aggregometers (see page 10).

The viscous medium used in the RW and AFRW deformability experiments was a 360 kDa polyvinylpyrrolidone (Sigma, 300 mOsm/kg, η=29.8 mPa·s in PBS) solution, while the ethanol study was performed with a 70 kDa dextran (Sigma, 297 mOsm/kg, η=28.4 mPa·s in PBS) solution. In both cases, the shear stress was varied, in steps, from 0.3 to 30 Pa. Measurements were performed at 37°C.

For some deformability results, EI-SS data was fitted to a Lineweaver-Burke type non-linear equation that yields the maximum EI at infinite shear stress (EI_{max}) and the stress required to achieve one-half of this maximum value ($SS_{1/2}$) [88, 89]. For the more accurate plot fitting, negative deformability values were excluded from the analysis. Data fitting and analysis were carried out using non-linear regression (GraphPad Prism, GraphPad Software, La Jolla, CA).

Throughout the RBC aggregometry experiments, temperature was kept at 37°C for LORCA measurements, while Myrenne was operated at ambient temperature.

2.4. Red blood cell suspensions

Red wine and blood samples were initially mixed to simulate final blood alcohol concentration of 0.10, 0.30 and 1%, while other samples were treated with AFRW or PS in a similar manner. Samples were incubated for 1 hour at ambient temperature on a rollerbed followed by RBC deformability and aggregation measurements.

In a 2nd study, two general approaches were utilized to evaluate the ethanol effects on erythrocyte deformability: 1) direct addition of ethanol to whole blood followed by incubation and testing; 2) addition of ethanol *only* to the viscous suspending medium used for deformability measurements. In the direct addition studies, ethanol was added to whole blood to achieve final concentrations of 0, 0.25, 0.50, 1 and 2%, following which these samples were incubated at room temperature for 1 hour then studied. In the other approach, alcohol was added directly to the viscous medium used for ektacytometry measurements at concentrations of 0, 0.25, 0.50, 1, 2, 3, 4, 5 and 6%, following which untreated red blood cells were suspended in these media then measured.

Aggregation measurements used 40% hematocrit suspensions of erythrocytes in autologous plasma or in a 70 kDa dextran solution (3% in PBS). In these studies, red blood cells were initially suspended in plasma at a 40% hematocrit, ethanol added at concentrations of 0, 0.25, 0.50, 1 and 2%, and incubated for 1 hour at room temperature. Erythrocyte-plasma samples were then tested without further processing, while cells to be suspended in 3% 70 kDa dextran were washed twice with PBS then re-suspended in the dextran at 40% hematocrit.

In a 3rd experiment, *in vitro* effect of red wine, alcohol-free red wine extract and ethanol was examined in the presence of oxidative stress. Blood samples were pretreated with RW and AFRW at a concentration of 0.30% or ethanol at concentrations of 0, 0.25, 0.50, 1 and 2% and then the free radical generator phenazine methosulfate (PMS, Sigma) was added at a final concentration of 500 μM. Samples were incubated at 37^{o}C for 2 hours then erythrocyte deformability was tested. In one series, whole blood was treated only with PMS and alcohol added only to the viscous medium used for deformability measurements.

2.5. Miscellaneous

After ethanol treatment, RBC shape was evaluated by DIC light microscopy (model BX50F; Olympus, Tokyo, Japan).

For statistical analysis paired t-tests were used to test changes from control (i.e., PS or PBS treated samples) with significance accepted at $p<0.05$.

The detailed technical differences in methodology between the red wine and ethanol studies (i.e., blood sampling, suspending medium of LORCA, etc) originate in the different possibilities of the two hemorheological laboratories in which the experiments were done.

3. Results

3.1. Results of the deformability measurements

Our results show that no erythrocyte deformability changes were observed in any concentrations of the red wine and alcohol-free red wine extract treated samples followed by incubation then testing with LORCA (Fig. 3A). Analysis obtained using the Lineweaver-Burke regression indicated that in case of the two agents neither EI_{max}, $SS_{1/2}$ nor their ratio differed from the saline treated control (Fig. 3B, C and D).

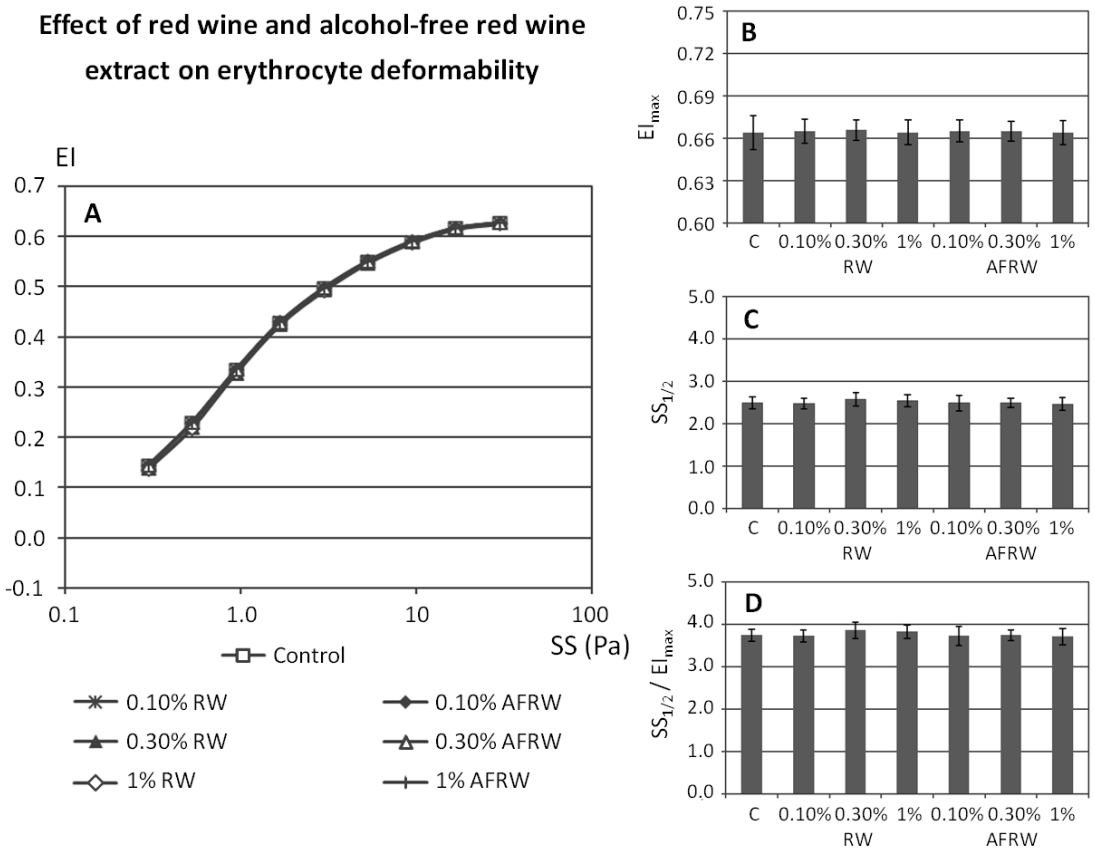

Fig. 3. A) *Erythrocyte deformation as an elongation index (EI) versus shear stress (SS) when red wine (RW) or alcohol-free red wine extract (AFRW) (0-1%) were added to whole blood followed by incubation and testing with LORCA.* ***B)*** *EI_{max},* ***C)*** *$SS_{1/2}$ and* ***D)*** *$SS_{1/2}$ / EI_{max} calculated using non-linear regression of the Lineweaver-Burke equation. Control (C) means physiological saline treated samples. N=13, values are mean ± SD. No significant differences were detected.*

The effects of ethanol on red blood cell deformability depended on the manner in which cells were exposed to the alcohol: 1) addition to whole blood followed by incubation caused no change in deformability (Fig. 4A); 2) addition to the LORCA media and testing of non-incubated cells resulted in significant, dose-dependent deformability increase ($p<0.05$) (Fig. 5A). No changes were observed in $EI_{max,}$ $SS_{1/2}$ and $SS_{1/2}$ / EI_{max} for incubated cells (Fig. 4B, C and D). Conversely, EI_{max}, $SS_{1/2}$ and their ratio for non-incubated cells significantly decreased ($p<0.05$) with alcohol concentration of the LORCA media (Fig. 5B, C and D). Note, however, that the magnitude of these alcohol-induced changes differed greatly: at 6% ethanol (the highest concentration studied), EI_{max} was only 1% below control whereas $SS_{1/2}$ and the $SS_{1/2}$ / EI_{max} ratio decreased by 46% and 43%. Thus, there was essentially no meaningful change of EI_{max} whereas the shear stress needed to achieve one-half of EI_{max} was markedly reduced.

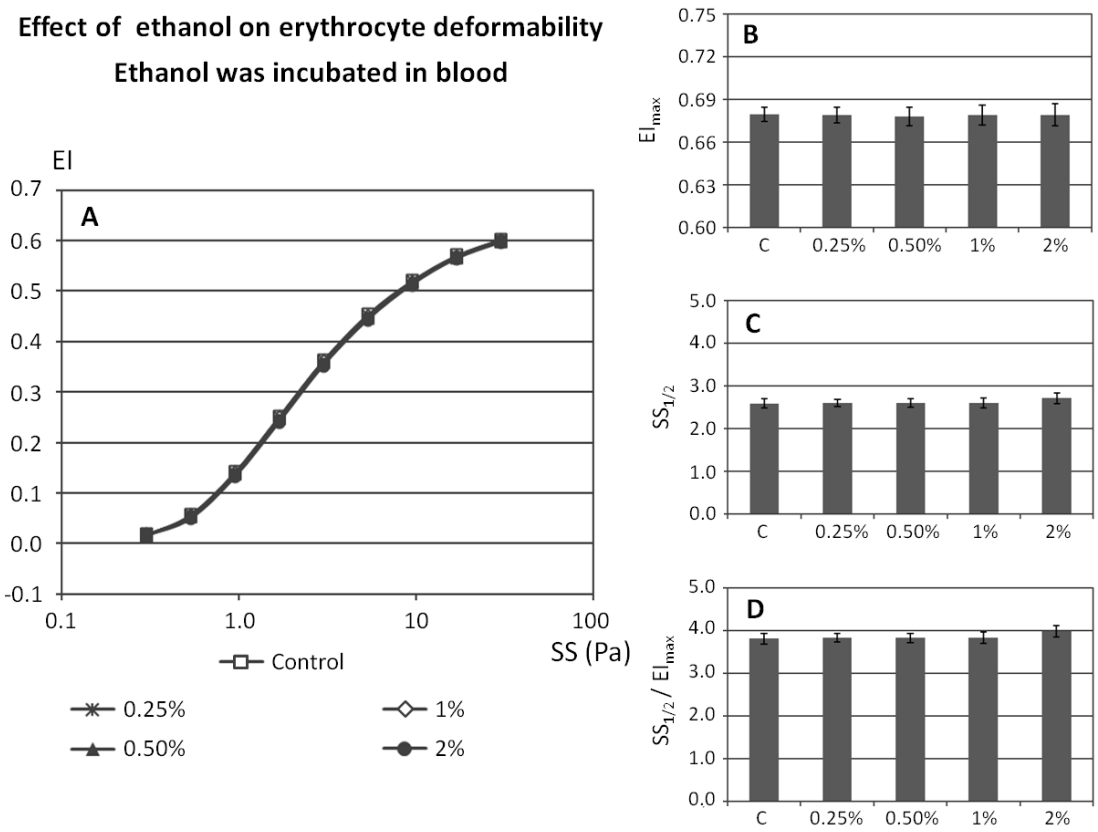

Fig. 4. A) *Erythrocyte deformation as an elongation index (EI) versus shear stress (SS) when ethanol (0-2%) was added to whole blood.* ***B)*** *EI_{max},* ***C)*** *$SS_{1/2}$ and* ***D)*** *$SS_{1/2}$ / EI_{max} calculated using non-linear regression of the Lineweaver-Burke equation. Control (C) means phosphate buffered saline treated samples. N=7, values are mean ± SD. No significant differences were detected.*

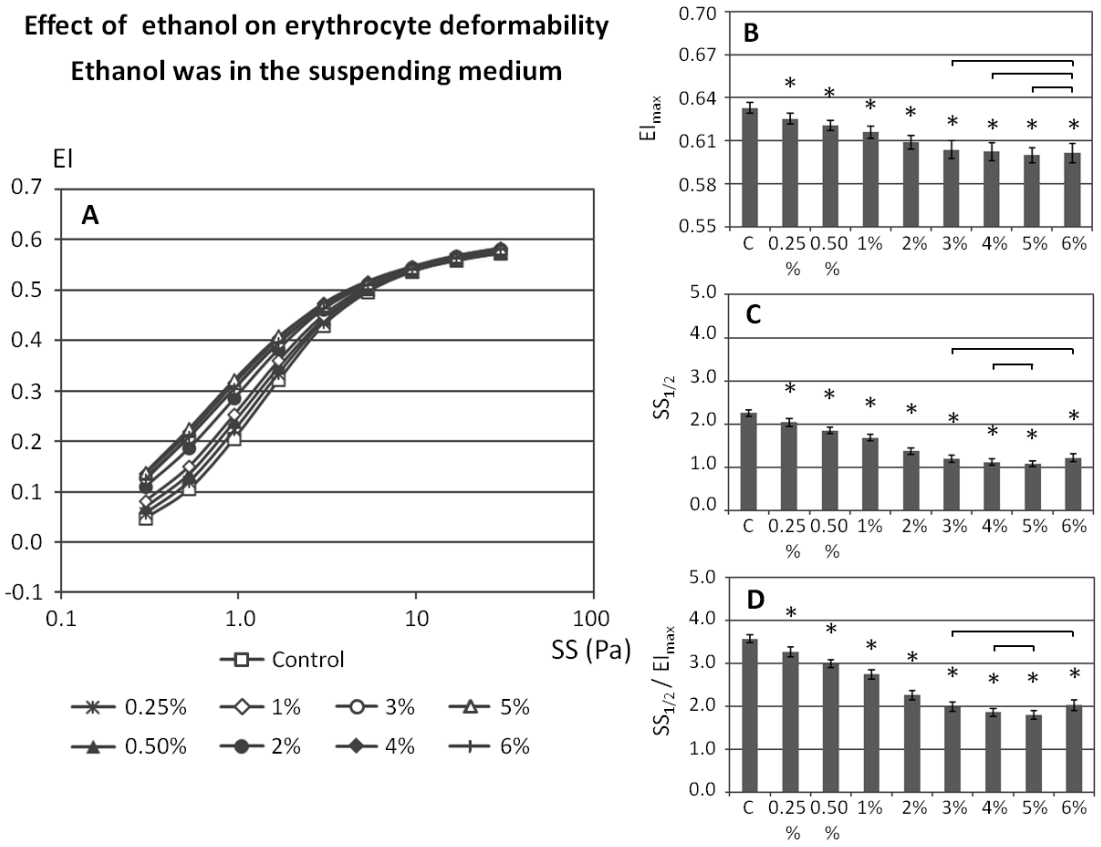

Fig. 5. A) *Erythrocyte deformation as an elongation index (EI) versus shear stress (SS) when ethanol (0-6%) was added to the suspending medium of the LORCA ektacytometer; cells were not pre-incubated with ethanol.* ***B)*** EI_{max}, ***C)*** $SS_{1/2}$ *and* ***D)*** $SS_{1/2}$ / EI_{max} *calculated using non-linear regression of the Lineweaver-Burke equation. Control (C) means only a phosphate buffered saline dilution added to the LORCA media. N=7, values are mean ± SD. Stars represent significant differences from control samples at p<0.05, while links show differences which are not significant.*

3.2. Results of the oxidative stress experiment

The effects of RW and its major components on erythrocyte deformability when cells were oxidatively stressed by the free radical generator phenazine methosulfate were also studied using the LORCA ektacytometer.

As expected [90], incubation with 500 μM PMS alone caused a significant decrease ($p<0.05$) of erythrocyte deformability (Figs. 6, 7 and 8).

Although AFRW pretreatment at 0.30% concentration significantly prevented ($p<0.05$) erythrocytes from the PMS generated deformability impairment, 0.30% RW had no such effect (Fig. 6A). The Lineweaver-Burke analysis demonstrated that EI_{max} for AFRW plus PMS treated cells did not change, while $SS_{1/2}$ and the $SS_{1/2}$ / EI_{max} ratio significantly decreased ($p<0.05$) by ~17% from the only PMS treated samples. On the other hand, EI_{max}, $SS_{1/2}$ and their ratio showed no alterations for erythrocytes treated with 0.30% RW and PMS compared to the only PMS damaged cells (Fig. 6B, C and D).

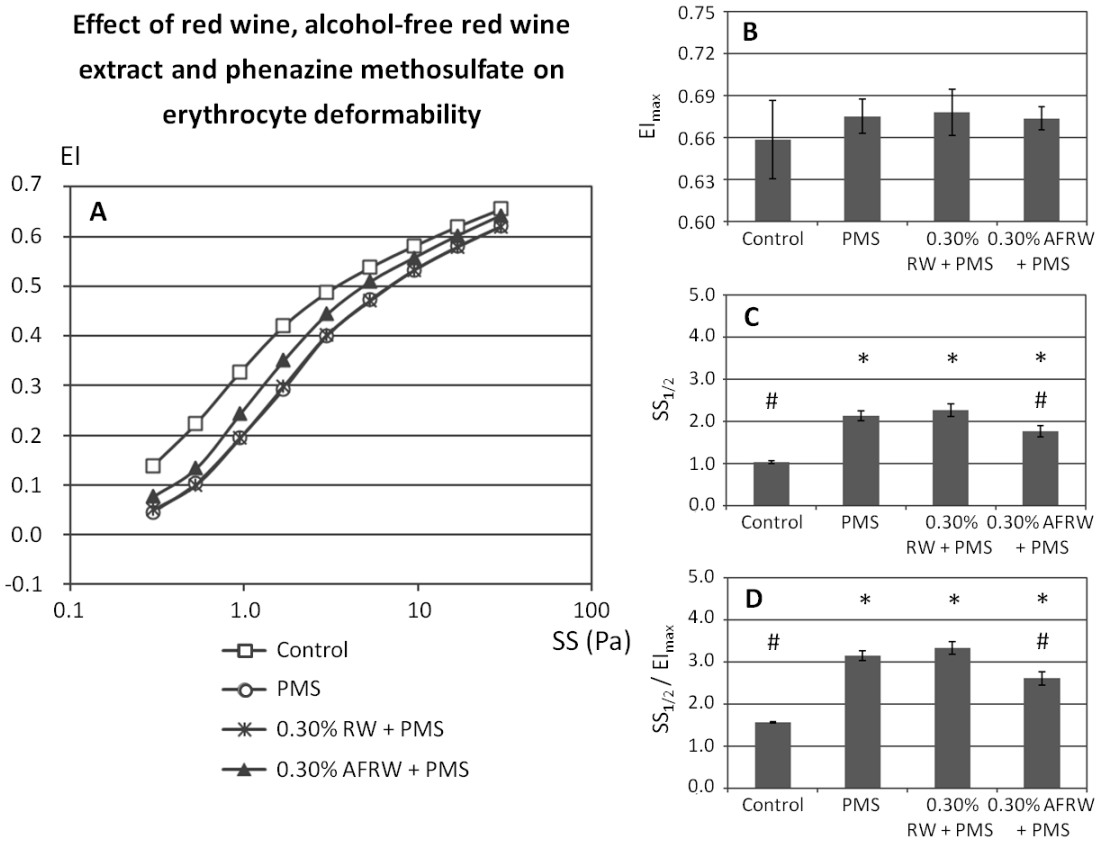

Fig. 6. A) *Erythrocyte deformation as an elongation index (EI) versus shear stress (SS) for whole blood incubated with 0.30% red wine (RW) or 0.30% alcohol-free red wine extract (AFRW) + 500 μM phenazine methosulfate (PMS); following incubation, cell deformability was measured with LORCA* ***B)*** *EI_{max},* ***C)*** *$SS_{1/2}$ and* ***D)*** *$SS_{1/2}$ / EI_{max} calculated using non-linear regression of the Lineweaver-Burke equation. Control means physiological saline treated samples. N=7, values are mean ± SD. Stars represent significant differences from control, while crosses show significant differences from PMS treated samples at p<0.05.*

Changes of erythrocyte deformability again depended strongly upon the manner in which cells were exposed to ethanol. Red blood cells incubated for 2 hours with alcohol plus PMS then tested in alcohol-free LORCA media exhibited significant decreases ($p<0.05$) of deformability from PMS alone (Fig. 7A). Under these conditions, EI_{max} for PMS treated cells was unaffected by the presence of ethanol during incubation, while both $SS_{1/2}$ and the $SS_{1/2}$ / EI_{max} ratio significantly increased ($p<0.05$) by ~20% at 2% ethanol (Fig. 7B, C and D).

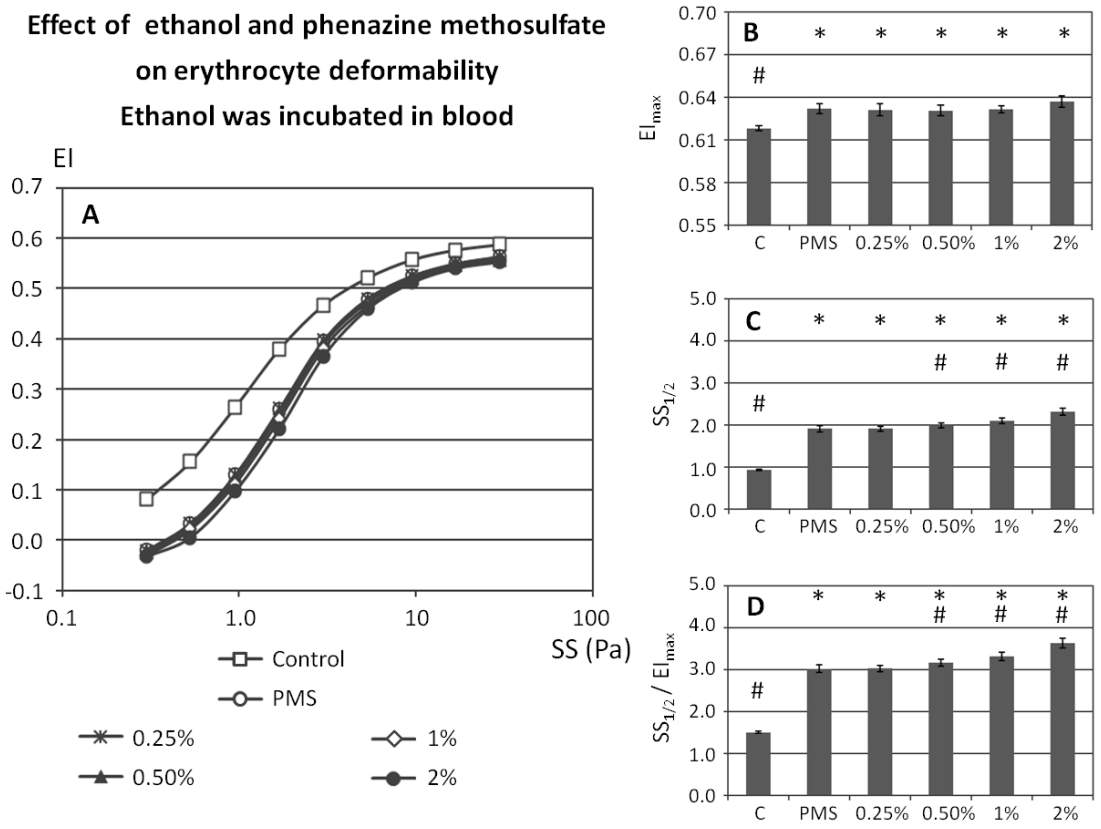

Fig. 7. A) *Erythrocyte deformation as an elongation index (EI) versus shear stress (SS) for whole blood incubated with ethanol (0-2%) + 500 μM phenazine methosulfate (PMS); following incubation the cells were tested in ethanol-free LORCA media.* ***B)*** *EI_{max},* ***C)*** *$SS_{1/2}$ and* ***D)*** *$SS_{1/2}$ / EI_{max} calculated using non-linear regression of the Lineweaver-Burke equation. Control (C) means phosphate buffered saline treated samples. N=7, values are mean ± SD. Stars represent significant differences from control, while crosses show significant differences from PMS treated samples at $p<0.05$.*

However, PMS treated cells tested with alcohol in the LORCA media exhibited significant improvements ($p<0.05$) of deformability compared to PMS alone (Fig. 8A), EI_{max} was not meaningfully altered, while $SS_{1/2}$ and the $SS_{1/2}$ / EI_{max} ratio were ~17% lower at 2% ethanol (Fig. 8B, C and D).

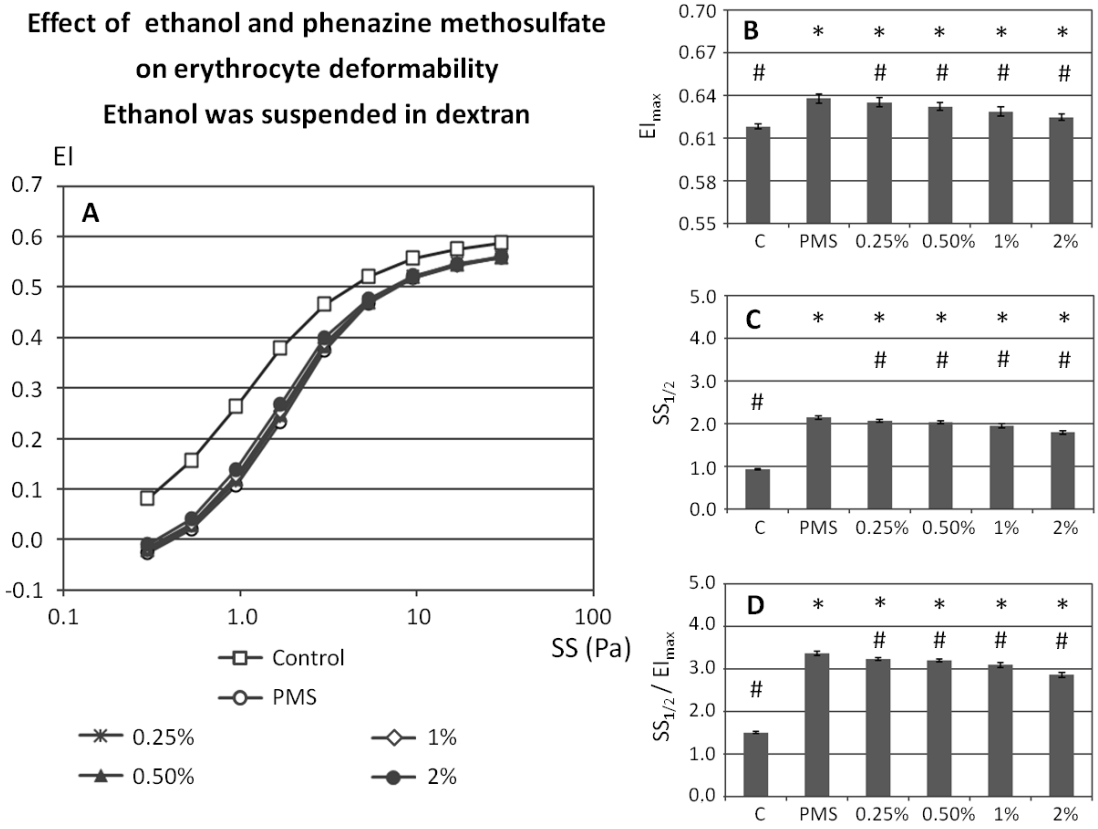

Fig. 8. A) *Erythrocyte deformation as an elongation index (EI) versus shear stress (SS) for whole blood incubated with 500 μM phenazine methosulfate (PMS); following incubation, cell deformability was measured in LORCA media containing 0-2% ethanol.* ***B)*** *EI_{max},* ***C)*** *$SS_{1/2}$ and* ***D)*** *$SS_{1/2}$ / EI_{max} calculated using non-linear regression of the Lineweaver-Burke equation. Control (C) means phosphate buffered saline treated samples. N=7, values are mean ± SD. Stars represent significant differences from control, while crosses show significant differences from PMS treated samples at $p<0.05$.*

3.3. Results of the aggregation measurements

Treatments with RW and AFRW inhibited erythrocyte aggregation in a dose dependent manner (Fig. 9). In M and M1 mode of the Myrenne aggregometer the differences were significant ($p<0.05$) already at a concentration of 0.10%.

Red wine had a tendency for stronger inhibition compared to AFRW which became significant ($p<0.05$) at a concentration of 1%: mean reductions in parameter M and M1 at highest concentration were 48% and 22% for AFRW as well as 80% and 43% for red wine (Fig. 9A).

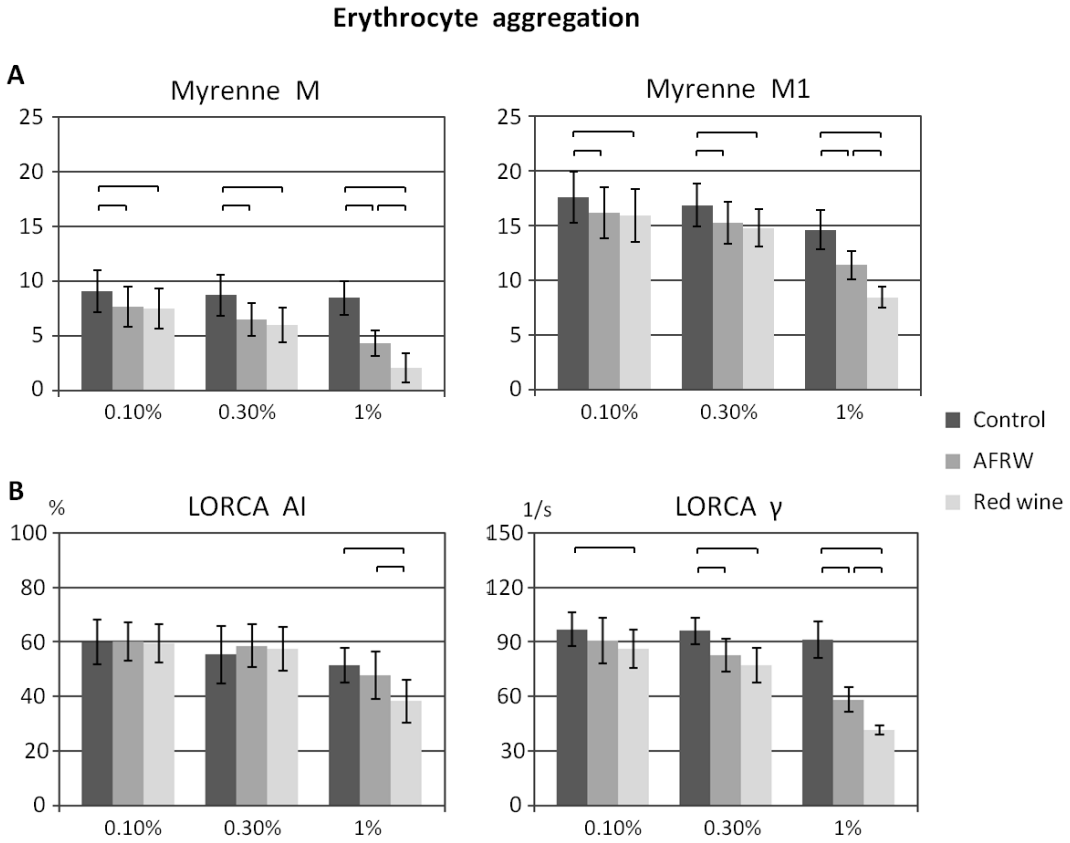

Fig. 9. *Erythrocyte aggregation determined by* ***A)*** *Myrenne showing its M and M1 parameters and* ***B)*** *Lorca aggregometer representing aggregation index (AI) and treshold shear rate (γ) after treatments with alcohol-free red wine extract (AFRW) and red wine (RW). Control means physiological saline treated samples. Effects of agents are compared within the same concentration group to eliminate the impact of sample dilution. N = 13, values are mean ± SD. Links represent significant difference at p<0.05.*

LORCA aggregation index (AI) confirmed these results only at the highest concentration where the difference between RW and AFRW became significant ($p<0.05$). Changes in LORCA threshold shear rate (γ) were concordant with Myrenne parameters: mean reductions in γ at the highest concentrations were 38% for AFRW and 55% for RW ($p<0.05$) (Fig. 9B).

Further results show that erythrocyte aggregation in autologous plasma (Fig. 10A) or in 3% 70 kDa dextran solution (Fig. 10B) showed significant decreases ($p<0.05$) in a dose-dependent manner after ethanol treatment. The changes of the M and M1 indices were significant at 0.25% and above, with the greatest decreases at 1% alcohol: mean reductions of M and M1 parameters were 24% and 18% for aggregation in plasma and 43% and 32% for aggregation in dextran. At 2% ethanol the aggregometer was unable to detect RBC aggregate formation in either medium.

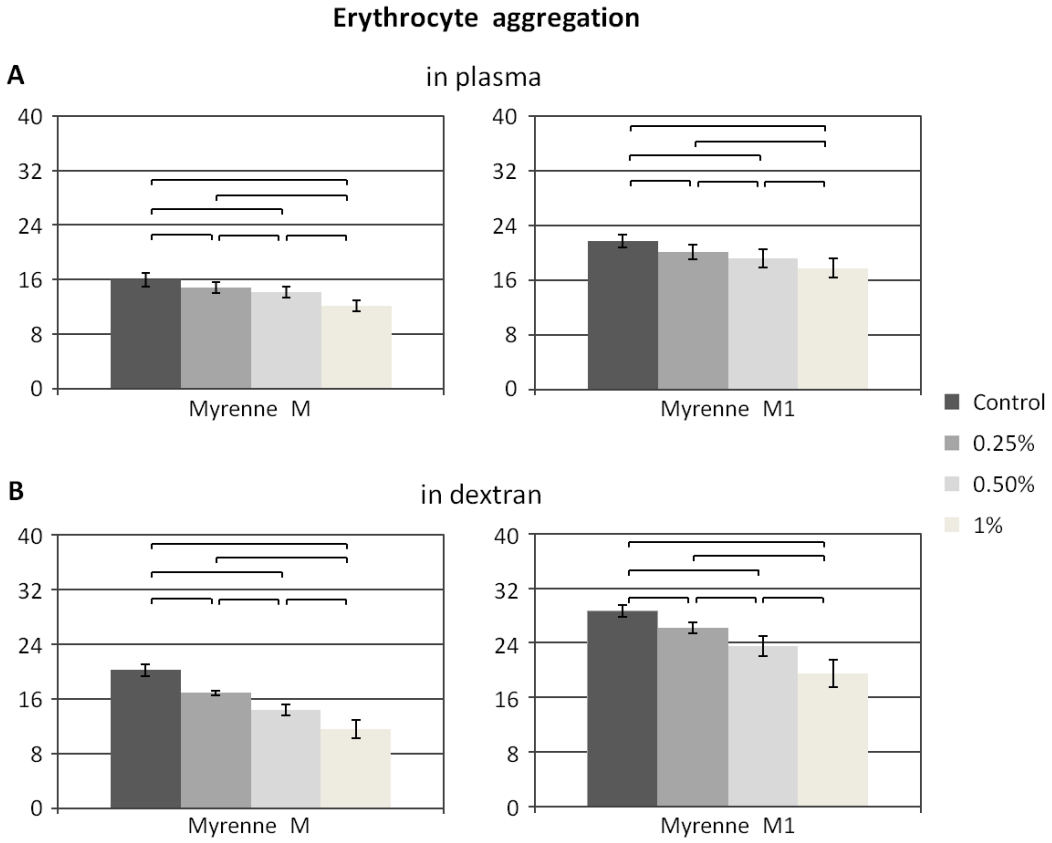

Fig. 10. *Erythrocyte aggregation determined by Myrenne aggregometer* ***A)*** *in autologous plasma or* ***B)*** *in 3% 70 kDa dextran solution. No aggregation was measureable at 2% concentration. Control means phosphate buffered saline treated samples. N=7, values are mean ± SD. Links represent significant differences at p<0.05.*

3.4. Microscopic analysis

Morphological analysis using DIC light microscopy demonstrated that normal discocytes (i.e., biconcave shaped cells) in PBS (Fig. 11A) became echinocytes (i.e., erythrocytes with spiky projections on the cell surface) with 2% ethanol (Fig. 11B). The viscous dextran medium used in the LORCA induces a slight stomatocytic (i.e., cup shaped red blood cells) transformation (Fig. 11C), while erythrocytes retain their normal, discocytic shape in dextran with 2% alcohol (Fig. 11D).

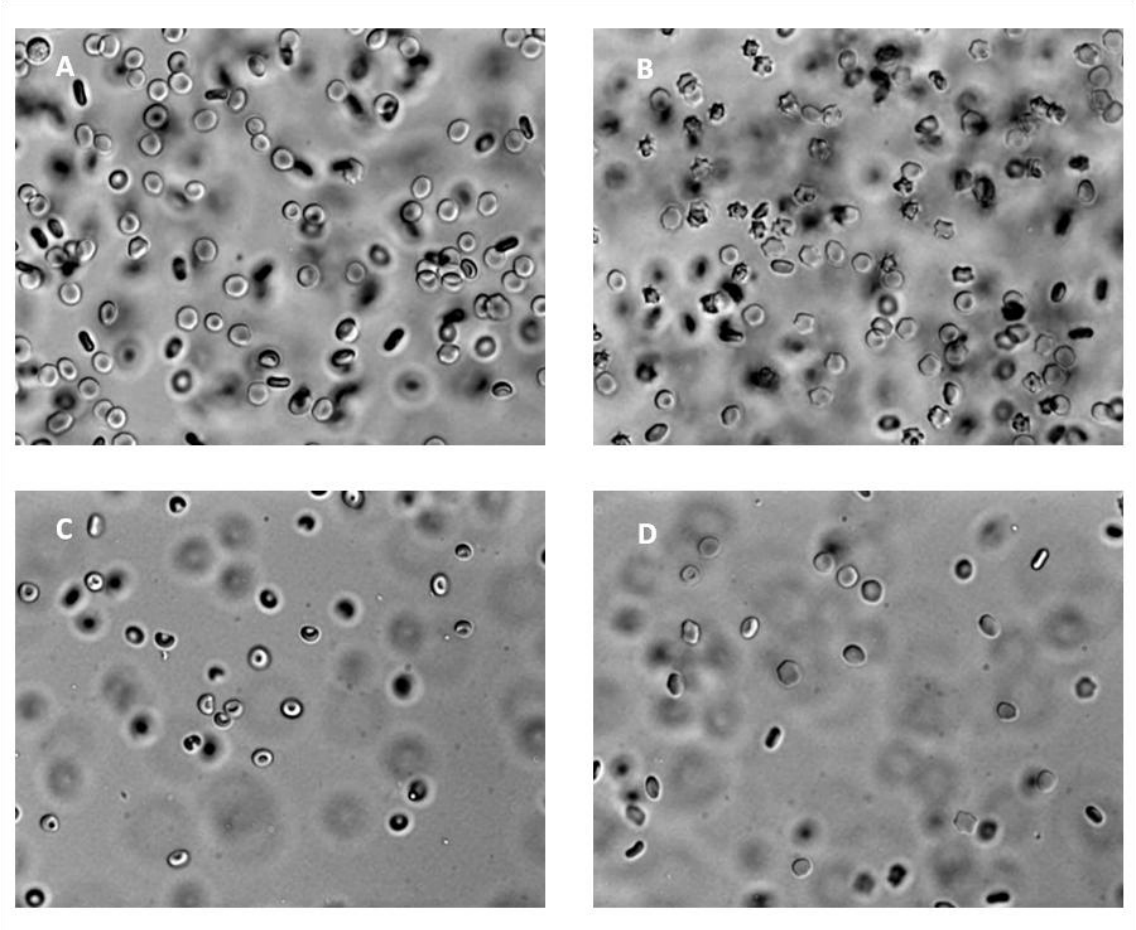

Fig. 11. *Morphological appearance of erythrocytes visualized by DIC light microscopy.* ***A)*** *Untreated erythrocytes in PBS demonstrating the normal, discoid shape of the cells.* ***B)*** *Echinocytes in PBS with 2% ethanol concentration.* ***C)*** *Stomatocytes in the viscous medium (dextran) of LORCA.* ***D)*** *Erythrocytes maintaining the normal, discocytic shape in dextran containing 2% ethanol.*

4. Discussion

Direct addition of red wine or alcohol-free red wine extract to blood is obviously not physiological. Furthermore, ethanol concentrations used herein greatly exceed levels (over 0.50%) that are physiologically tolerable. In many locations throughout the world, intoxication and inability to operate machinery (e.g., drive a car) is assumed at 0.08 to 0.1% ethanol. On the other hand, throughout the development process of a new pharmacological agent many different kinds of *in vitro* and *in vivo* investigations are made. In this *in vitro* study, blood was used as an isolated human tissue and the measurements with red wine and its major components may provide several reproducible pharmacodynamic effects. High, *in vivo* intolerable concentrations can be accepted in the setting of an *in vitro* investigation. Furthermore, measurements with high, intolerable alcohol concentrations can reveal valuable toxicological information.

In this *in vitro* experiment, hemorheological consequences of red wine and its major components were examined focusing on erythrocyte deformability measured by LORCA ektacytometer (see page 8) and aggregation determined by Myrenne and LORCA aggregometers (see page 10).

4.1. Analysis of the deformability results

Erythrocyte deformability and its determining factors have already been discussed (see page 5) [2, 9-11]. The relative importance of these parameters for altering deformability can depend on the testing system and the level of applied forces; abnormal deformation behavior may be detected at low stress level forces but may not be evident when much higher forces are applied. In order to avoid choosing the appropriate stress for comparisons, we have elected to utilize a curve fitting approach over the entire range of shear stress (i.e., 0.3-30 Pa) in order to characterize RBC mechanical behavior by just two parameters (i.e., EI_{max} and $SS_{1/2}$); this approach has been validated and shown to be appropriate for various erythrocyte populations [88, 89].

The results of our *in vitro* study indicate that direct addition of RW and AFRW to blood followed by incubation do not alter erythrocyte deformability, while ethanol can improve it when the cells are subjected to fluid stress in a defined shear field.

These improvements were *only* observed when ethanol was in the viscous media used for ektacytometry testing (Fig. 5) and were *not* present when cells were incubated with the alcohol but tested in alcohol-free viscous media (Fig. 4). Our *in vitro* results thus indicate that the ethanol-induced deformability improvement requires the presence of ethanol. These results were supported by several *in vivo* experiments [86, 91] and *in vitro* studies suggesting that changes in the cell membrane are reversible [92, 93].

Comparing our deformability results to literature reports is problematic since variety of methods was used. The effects of ethanol consumption depend on the drinking habits of the subjects tested. Using micropore filtration, studies have shown that erythrocyte deformability is reduced in active alcoholics [94, 95]. On the other hand, ektacytometry results have shown increased deformability at high shear stresses 1.5 hours after the ethanol intake [91], while a recent study indicates no changes after red wine or moderate vodka consumption for two weeks [86]. Prior *in vitro* studies are also not in concordance: filterability measurements have demonstrated increased RBC deformability at physiological concentrations of ethanol [96], while a micropipette aspiration technique has shown that high, intolerable levels of ethanol decreases deformability [97]. Our results indicate that the greatest enhancement of deformability, indexed by $SS_{1/2}$ and the $SS_{1/2}$ / EI_{max} ratio, was observed at 4% and 5% ethanol concentration, while deformability at 6% was significantly lower and similar to the 3% ethanol results (Fig. 5). These findings thus indicate a bi-phasic effect of ethanol: improved deformability followed by decreased benefits with increasing concentration.

As indicated above, four factors (e.g., morphology, geometry, membrane rheologic properties and cytoplasmic viscosity) can affect erythrocyte deformation behavior [9, 10]. Although ethanol can cause a discocyte-echinocyte shape change [93, 98], cells suspended in dextran + ethanol generally have a discoidal morphology (Fig. 11). Ingested alcohol increases plasma osmolality [99, 100], thereby reducing cell volume [80], increasing surface to volume ratio and elevating cytoplasmic viscosity; the increased ratio favors deformability, while the greater cytoplasmic viscosity has the opposite effect. Given that cells were always suspended in isotonic media, it thus seems most likely that ethanol affects the mechanical behavior of the membrane with its attached cytoskeleton. The importance of the cytoskeleton for the cell's physical behavior has been shown in a detailed analysis of RBC membrane properties [8].

Ethanol has a polar hydroxyl group soluble in aqueous media and hence must distribute within the exterior glycocalyx and the interior of the cell, while the non-polar part of the molecule is preferentially found in the lipid bilayer [83, 101]. The fluidity of the lipid portion can be altered by ethanol in a dose dependent manner: 1) up to 0.3% there is no change in the membrane's external layer (TMA-DPH fluorescence) or its hydrophobic region (DPH fluorescence) [79]; 2) increased fluidity up to 1.6% as assayed by electron paramagnetic resonance [101]. Note, that the less viscous lipid bilayer has only minimal influence on cell deformability [8, 10, 102]. Thus the cytoskeleton must be reversibly altered in a manner that decreases membrane shear modulus [103, 104]. The most likely molecular change is a weakening of spektrin-aktin linkages [8, 103]. In addition, it is possible that interactions between transmembrane proteins and cytoskeletal components are involved [10].

4.2. Analysis of the oxidative stress experiment

RBC deformability alterations induced by red wine, alcohol-free red wine extract and ethanol were also examined in the presence of oxidative stress generated by phenazine methosulfate. PMS is a well-known oxygen free radical generator that causes lipid peroxidation and structural modifications in the membrane skeletal protein network, leading to increased membrane rigidity and decreased deformability [90]. These harmful effects of PMS have successfully been employed in previous hemorheological studies using filtration technique where antioxidant effects of various cardio- and cerebrovascular drugs were investigated [105, 106].

Determination of erythrocyte deformability after RW or AFRW pretreatment demonstrated that AFRW significantly decreased the PMS generated RBC deformability impairment thus prevented erythrocytes from oxidative stress (i.e., decreased $SS_{1/2}$ and the $SS_{1/2}$ / EI_{max} ratio) (Fig. 6). Our *in vitro* results confirm prior *in vivo* results about the antioxidant properties of polyphenols where plasma antioxidant capacity was increased by the consumption of AFRW in human volunteers [107]. Although AFRW could partially protect erythrocytes, red wine pretreatment had no such preventive influence in this model.

This red wine observation is presumably supported by the *in vitro* ethanol experiment, where pure ethanol + PMS were added together to whole blood followed by incubation then tested in alcohol-free LORCA media (Fig. 7); ethanol enhanced the effect of oxidative stress with increasing concentration leading to progressively decreased erythrocyte deformability compared to blood samples containing only PMS treatment (i.e., increased $SS_{1/2}$ and the $SS_{1/2}$ / EI_{max} ratio). Based on these results it seems reasonable that the protective effect of polyphenols is attenuated by the presence of ethanol in the red wine portion.

On the other hand, the ethanol + PMS results also showed that the deformability of oxidatively damaged erythrocytes could be improved when ethanol was present in the LORCA media (Fig. 8). However, the deformability improvement was not that remarkable, ethanol presumably acts in a manner similar to the effects on normal erythrocytes (Fig. 5). Furthermore, this observed difference in the magnitude of the alcohol-induced deformability improvements (i.e., with vs. without PMS) confirms the above mentioned speculations about the modifications in the viscoelasticity of the cell membrane, since PMS impairs the lipid components of the membrane as well as the attached cytoskeletal protein network, while ethanol supposedly has the opposite effect; increases the fluidity of the lipid layers and alters the organization between transmembrane and cytoskeletal proteins.

4.3. Analysis of the aggregation results

There are multiple factors that can characterize RBC aggregation which is explained by two parallel models: 1) the bridging theory; 2) the depletion layer model (see page 6) [18-20].

In this experiment, red wine and alcohol-free red wine extract were incubated with whole blood then tested with Myrenne and LORCA aggregometers demonstrating a dose-dependent reduction in erythrocyte aggregation and indicating that RW is a more potent inhibitor of RBC aggregation than AFRW (Fig. 9).

This decrease may be a consequence of the changes in RBC membrane and in plasma components especially modifications of plasma proteins. Polyphenols can bind to plasma proteins due to their poor water solubility. Based on the bridging theory for aggregation, the phenol-protein interactions presumably alter the properties of proteins leading to reduced capability to form cross links between cellular components leading to decreased erythrocyte aggregation.

The alcohol experiment is consistent with a prior report indicating decreased aggregation when ethanol is added to whole blood (Fig. 10A) [79] and also demonstrates that aggregation is reduced when this alcohol is added to a suspension of erythrocytes in 3% 70 kDa dextran (Fig. 10B). Furthermore, this experiment gave the explanation why RW and not AFRW showed the greater inhibitory effect on RBC aggregation. Decreased aggregation in plasma may be partially due to the ethanol-induced echinocytic shape transformation (Fig. 11) [98] and to alteration or destruction of plasma proteins that promote aggregation (e.g., fibrinogen). Reduced RBC deformability also tends to reduce aggregation [20]; however, our results indicate an increased cellular deformability (Fig. 2). It therefore seems most likely that ethanol-induced changes of the RBC glycocalyx are involved. Based on the depletion layer model for aggregation, the scale of a protein or polymer depletion zone near the membrane depends strongly on the ability of the macromolecule to penetrate the glycocalyx [19, 20]; increased penetration would reduce aggregation.

It is interesting to note, that this presumed change of glycocalyx properties is irreversible, since reduced RBC aggregation was observed for cells incubated with ethanol but suspended in ethanol-free dextran (Fig. 10B).

5. Conclusion

In summary, these *in vitro* measurements indicate that red wine, alcohol-free red wine extract and ethanol have some effects on hemorheological parameters including erythrocyte deformability and aggregation.

Our investigations proved that AFRW can protect erythrocytes and preserve their deformability from oxidative stress mediated impairment, while RW had no such effect (Fig. 6). Both RW and AFRW reduce RBC aggregation although RW is the more potent inhibitor (Fig. 9). Furthermore, ethanol reversibly improves erythrocyte deformability and irreversibly decreases RBC aggregation (Figs. 5 and 10). The presence of ethanol in blood enhances the oxidative stress induced RBC deformability impairment and improves the deformability of the previously damaged cells (Figs. 7 and 8).

It is important to note that the cardiovascular risk reduction associated with moderate red wine drinking is most likely related to the combined beneficial effects of red wine components (e.g., different polyphenols, ethanol): separate studies of these main components may not reflect the overall response seen with red wine. In our opinion, the found beneficial hemorheological changes (i.e., improved RBC deformability and decreased aggregation) enhance the tissue perfusion and may play a role in the cardiovascular protective effects of moderate red wine consumption.

Although our preliminary results demonstrate that moderate red wine consumption has some beneficial effects on hemorheological parameters, additional studies are obviously needed to prove these findings under *in vivo* circumstances. The specific molecular mechanisms involved also require further investigations. Identification of the most important polyphenolic components of red wine and investigation of their specific effect in pharmacological doses may also be interesting for future research.

VI. Analysis of light scattering by red blood cells in ektacytometry

1. Introduction

1.1. Deformability of sickled erythrocytes

As it has been mentioned before in a more-detailed description, RBC deformability is the ability of erythrocytes to deform in response to mechanical forces which is essentially required for traversing capillaries and determined by several factors (e.g., morphology, geometry, membrane rheologic properties and cytoplasmic viscosity) (see page 5) [2].

Pathological red blood cell deformability can be seen in several disease states especially in sickle cell disease (SCD) characterized as a genetic disorder due to an amino acid substitution (valine for glutamic acid) at the 6^{th} position in the β-globin chain forming hemoglobin S (HbSS).

At low oxygen tension HbSS starts polymerizing leading to increased intracellular viscosity and diminished erythrocyte deformability with the typical distorted and elongated cell shape (the process is also termed sickling). Blood of patient with SCD contains different sub-populations of erythrocytes including normal, well-deforming discocytes, fairly rigid sickled cells (these reversibly sickled cells (RSC) can regain the discoid shape at high oxygen tension) and not deformable erythrocytes (irreversibly sickled cells (ISC) with no ability to recover the normal shape upon oxygenation).

Based on the general agreement, irreversibly sickled erythrocytes can be recognized with microscopic analysis because their length is twice as much as their width (Fig. 12). These rigid cells are fragile causing continuous hemolysis and anemia. Furthermore, sickled cells are also responsible for other main symptoms of SCD including capillary occlusions, painful crisis (i.e., pain due to impaired and insufficient tissue perfusion), infarctions of different organs and increased blood flow resistance in the lungs [12, 108, 109].

This chapter is based on the following article:
- Rabai M, Meiselman HJ, Wenby RB, Detterich JA, Feinberg J. Analysis of light scattering by red blood cells in ektacytometry using global pattern fitting. *Biorheol*, accepted for publication.

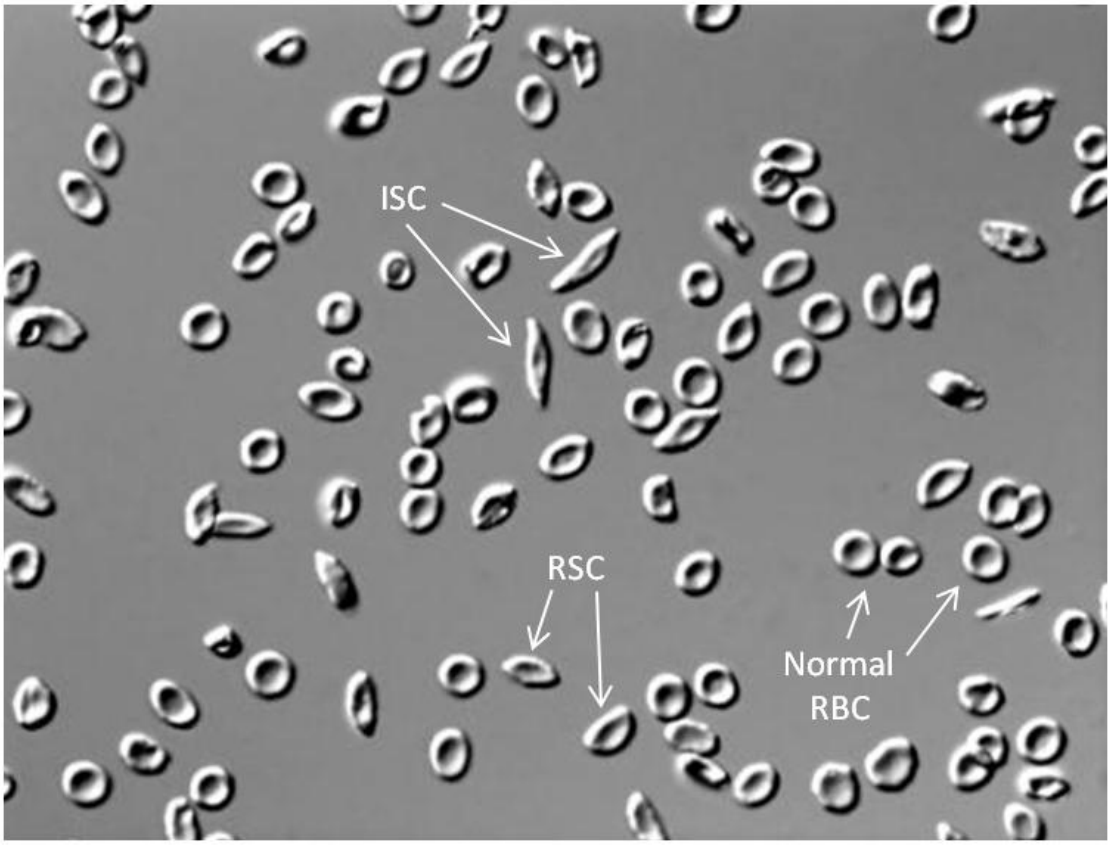

Fig.12. *Different shapes of erythrocytes (i.e., normal RBC, reversibly (RSC) and irreversibly (ISC) distorted sickled cells) in blood obtained from a patient with sickle cell disease.*

1.2. Previous analysis of diffraction patterns

Various methodological approaches of red blood cell deformability have been developed (e.g., filtration models, micropipette aspiration techniques and ektacytometry) (see page 8) [26-30].

The technique of ektacytometry (also known as laser diffraction ellipsometry) has already been described in detail (see page 8). In brief, it analyzes the laser diffraction patterns of red blood cells subjected to shear stress while suspended in a fluid. At low shear stress the essentially circular cells generate a circular diffraction pattern, while the increasing stress forces the cells to progressively deform into ellipsoidal shapes aligned with the flow, and thereby generate elliptical diffraction patterns (Fig. 13) [102, 110].

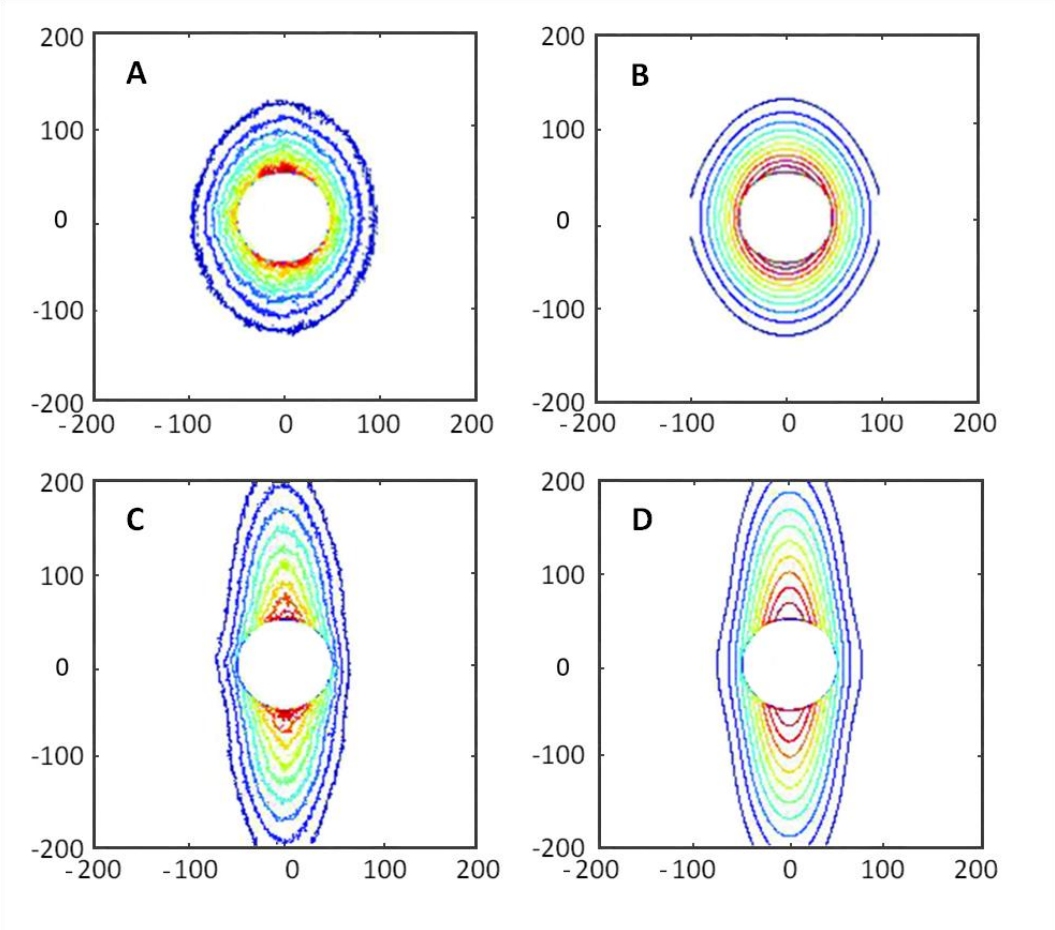

Fig. 13. *Contour plots of intensity diffraction patterns of a mixture of 100% normal red blood cells. Low shear stress (0.5 Pa):* ***A)*** *Observed diffraction pattern.* ***B)*** *Best fit of that diffraction pattern using a Bessel function. High shear stress (50 Pa):* ***C)*** *Observed diffraction pattern.* ***D)*** *Best fit of that diffraction pattern using an anomalous diffraction function.*

For analyzing the laser diffraction patterns, ektacytometry takes a single level slice through the measured laser intensity pattern and fits the resulting contour to an ellipse. If the major and minor axes of the fitted ellipse have lengths "*a*" and "*b*" respectively, then for each shear stress an elongation index; EI = (*a* - *b*) / (*a* + *b*) can be assigned to the cells.

However, in patients with sickle cell disease, the red blood cells are a mixture of normal cells together with a sub-population of poorly deformable sickle cells [111]. With such blood, the resulting laser diffraction pattern is a weighted average of the diffraction pattern of rigid discs (or non-deforming cells not aligned with the flow) together with the normally-deforming cells [112, 113].

Under increasing shear stress, normal cells progressively deform and yield elliptical diffraction curves, while the poorly deformable cells exhibit rigid body rotation and consequently produce an essentially circular diffraction pattern. The combined diffraction pattern of these two kinds of cells has a cross-like appearance; it is a distorted ellipse with a bump or bulge at its center (Fig. 14) [114, 115]. Accordingly, applying the commercial ellipse-fitting routines to such patterns yields incorrect values for the elongation index.

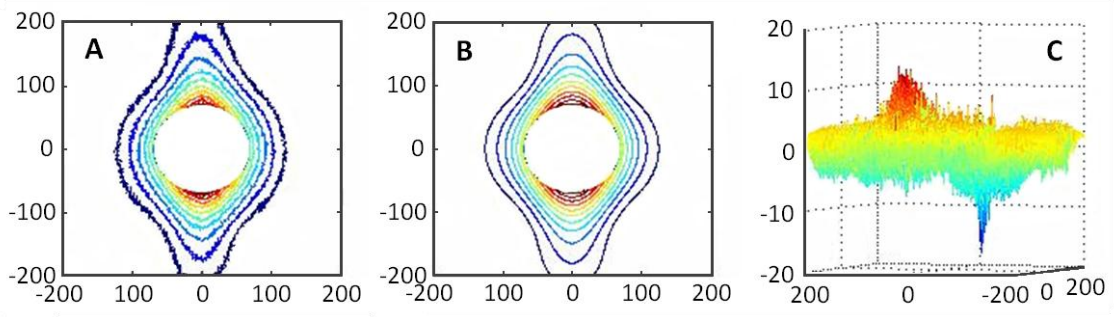

Fig. 14. *Contour plots of intensity diffraction patterns of a mixture of 70% normal + 30% rigid red blood cells.* ***A)*** *Measured diffraction pattern at high shear stress (50 Pa).* ***B)*** *Best fit to the measured diffraction pattern.* ***C)*** *Digital difference between the two patterns. Note the pronounced bumps in the center of the diffraction patterns due to the presence of the rigid cells.*

Streekstra and co-workers [115] analyzed such distorted diffraction patterns by considering mixtures of oblate and prolate spheroids and employing the anomalous diffraction approximation for spheroids. They were able to theoretically generate diffraction patterns for various mixtures and then apply their method to deduce the relative concentrations of rigid and deformable human red blood cells.

While aiding in the understanding of such distorted patterns, their approach was limited to a single, high-shear stress region (60 Pa) and mixtures of discoidal plus maximally deformed cells, and consequently did not extract the EI values for the deformable cells over the wide range of stress levels used in red blood cell ektacytometry [116].

In this study, a new method is described to analyze the diffraction patterns produced by a sickle cell blood model; a mixture of normal and abnormal red blood cells. The method relies on global curve fitting, in which a series of diffraction patterns taken at different shear stresses are analyzed simultaneously using a subset of fitting parameters common to all of the curves. The technique can reveal the elongation index of the normal cells even in the presence of non-deformable cells. Additionally, the method can estimate the fraction of non-deformable cells present in the blood.

2. Theory

An incident laser beam diffracts from the cell and travels to a distant screen whose *x-y* plane is perpendicular to the direction of the incident laser beam and is located at a distance *z* from the scattering cell. The intensity of the diffraction pattern observed on the screen is [117, 118]:

$$I\left(x,y,z\right)=\left(\frac{I_0}{k^2r^2}\right)\left|S(x,y,z)\right|^2 \qquad \text{Eq. 1}$$

Here I_o is the incident laser intensity, $r = (x^2 + y^2 + z^2)^{1/2}$ is the distance from the red blood cell to any point (x, y, z) on the viewing screen, and k is the wavevector of the laser light in air.

The scattering function $S(x, y, z)$ depends on the shape of the cell, and is calculated by first determining the amplitude of the incident light wave in a plane P' immediately after the cell and then propagating that transmitted wave from the plane P' to the plane of the viewing screen. If the light incident on the cell has a flat wavefront and if the screen is sufficiently far from the cell, the wave's propagation is described by a Fraunhofer diffraction integral [118].

The far-field diffraction pattern produced by erythrocytes depends on the shape of the cells, which varies with shear stress. At zero shear stress a RBC is a biconcave disc. It is assumed, that *at low shear stress* a normal RBC transforms from a biconcave disc into an elliptical disc having major and minor diameters *a* and *b* respectively, and having a uniform thickness, *c* (resembling a stretched hockey puck). In this case, the scattering function can be evaluated using Bessel function of the first kind [119].

It is assumed, that *at high shear stress* the red blood cell no longer resembles a disc of a uniform thickness and instead is modeled by an ellipsoid with axis diameters $a > b > c$. The resulting scattering function is a so-called "anomalous" diffraction pattern [117, 118, 120, 121].

On the other hand, combinations of cells create sums of diffraction patterns. In general, when a laser beam passes through a sample containing both rigid and deformable cells, the observed laser diffraction pattern is the incoherent sum of two scattered light waves [122], the first from the deformable cells and the second from the rigid, non-deformable cells. In the present study, a least-squares fit of this composite calculated function was performed to the *entire* measured diffraction pattern. It is assumed, that the rigid cells remain transparent discs of fixed size for all values of shear stress, and so the amplitude of their diffraction pattern $S(x, y, z)$ will be a Bessel-function pattern. Normal cells at low to moderate shear stress (<3 Pa) are discs, but at high shear stress (>3 Pa) become ellipsoids.

3. Global computer fits of observed diffraction patterns

At low values of the shear stress, both the normal and the rigid cells are described as discs of uniform thickness, and the fitting Bessel-function is proportional to the projected shadow area of the normal and of the rigid cells. In this case, there are seven adjustable parameters as well:

q1 - concentration of normal red blood cells
q2 - mean diameter of normal red blood cells
q3 - ratio of minor to major axes of normal red blood cells
q4 - concentration of rigid red blood cells
q5 - mean diameter of rigid red blood cells
q6 - ratio of minor to major axes of rigid red blood cells
q7 - uniform background of the photodetector

However, since the volume of a red blood cell as well as its surface area do not change as the cell is stretched, the thickness of the red blood cell can be computed if the cell's eccentricity and mean diameter are known; ~100 μm^3 [123] and ~140 μm^2 [124] for the fixed values of the cell's volume and surface area were used, respectively.

At high values of the shear stress, the normal cells are treated as ellipsoids, while the rigid cells remain discs. The form of the fitting function is then a mixture of "anomalous" and Bessel functions, so the intensity of the light passing through the normal cells is determined by not only the length of minor and major axes but by the thickness of the cell as well, which changes with applied shear stress. However, as in the case of the discs, the area and the volume of the cell remain constant under shear, so the thickness *c* can be calculated from the values of the other two dimensions of the ellipsoid.

In principle, five of the above seven parameters should not vary with shear stress. In particular, the concentration of normal cells (q1) as well as the background counts of the photodetector (q7) should not vary with shear stress. Similarly, for the rigid red blood cells the concentration (q4), mean diameter (q5), and axes ratio (q6) should all stay fixed as the shear stress is varied. This condition was employed by requiring that all of the fitting parameters except for the mean diameter (q2) and the axes ratio (q3) of normal cells maintain fixed values for *all* shear stress, and then minimize the *global* sum of the least-squares differences for *all nine shear stresses simultaneously*. Thus, the minimized following global sum over all nine shear stresses:

$$Global\ Sum = \sum_{\substack{shear\ stresses \\ j=1}}^{9} \sum_{\substack{all\ pixels \\ (x,y)}} \left[I^{j}{}_{measured}(x,y) - I^{j}{}_{computed}(x,y) \right]^2 \qquad \text{Eq. 2}$$

Only the two parameters, (q2) and (q3) are allowed to vary as the shear stress varies; the other five parameters are locked at their optimum values as determined by the computer. In effect, this method performed 23 parameter fit for all of the nine shear stress patterns simultaneously. Although somewhat time consuming, this approach yielded very well reproducible parameters.

4. Methods

4.1. Normal and rigid blood samples

Venous blood samples were obtained from healthy adult subjects; the study was approved by the Human Subjects Institutional Review Board, University of Southern California, Los Angeles, CA.

Tourniquet was applied to locate the antecubital vein prior to venipuncture and was released at the start of sampling that was completed within 90 seconds; the samples were anticoagulated with ethylenediamine-tetraacetic acid (1.5 mg/ml). Blood samples were centrifuged at 1,400 x g for 5 minutes. The plasma and the white cell layer were removed and discarded. Erythrocytes were washed twice with phosphate buffered saline (PBS, 290 mOsm/kg, pH = 7.4) then re-suspended in PBS.

Rigid, non-deformable erythrocytes were prepared by treating with glutaraldehyde, a di-aldehyde that reacts quickly with amino groups. A dilute red blood cell/phosphate buffered saline suspension was carefully added to an equal volume of 1% glutaraldehyde (Sigma Chemical Co., St Louis, MO, USA) in PBS followed by gentle stirring for 60 minutes at room temperature. Rigid cells were washed to remove any unreacted glutaraldehyde, then re-suspended in phosphate buffered saline and stored at 4°C until use. At the concentration employed the glutaraldehyde did not alter RBC volume or shape; light microscopy indicated that the rigid cells maintained the usual discoidal biconcave morphology.

4.2. Mixtures of normal and rigid cells

Mixtures of normal and rigid erythrocytes were prepared containing 0, 5, 10, 20, 30 and 50% rigid cells. The experimental protocol involved adjusting the normal red blood cell/phosphate buffered saline suspensions to a cell concentration equal to the rigid cell suspension using an automated hematology analyzer (Micros, Horiba-ABX, Irvine, CA, USA) to determine cell concentrations. Samples containing various proportions of rigid cells were prepared using appropriate volumes of the normal and rigid red blood cell suspensions while keeping the total cell concentration constant.

4.3. Erythrocyte deformability testing

The above mentioned global-fitting approach was tested with a LORCA ektacytometer (Laser Assisted Optical Rotational Cell Analyzer; R&R Mechatronics, Hoorn, Netherlands) measuring erythrocyte deformability of rigid and normal RBC mixtures. These different cell populations were added directly to a viscous, isotonic 70 kDa dextran solution (Sigma, 297 mOsm/kg, η=31.4 mPa.s in PBS), and mixed well to obtain a uniform suspension before being measured.

Throughout the measurements, diffraction patterns of the deforming erythrocytes were captured by a video camera and digitally stored. The central region of the diffraction pattern also contains the undiffracted laser spot and so is very bright; it is physically blocked by an opaque dot affixed to the viewing screen leading to a "hole" in the middle of the diffraction pattern (Figs. 13 and 14). For each sample, 10 patterns were digitally averaged at each shear stress and used for further processing of global parameter fitting.

5. Results and Discussion

5.1. Diffraction patterns of normal red blood cells

Throughout the erythrocyte deformability measurements, nine different shear stresses from 0.5 Pa to 50 Pa were used for the global fit for each RBC sample, but only the lowest and the highest shear stresses of the laser diffraction patterns generated by normal erythrocytes (0% rigid cells) are shown (Fig. 13). The seven fitting parameters (qi) are varied to minimize simultaneously the least-square difference between the nine measured diffraction patterns and the calculated patterns.

5.2. Diffraction patterns of a mixture of normal and rigid cells

Data of red blood cell samples containing 30% rigid and 70% normal erythrocytes are demonstrated as well (Fig. 14).

Note, that at high shear stress the measured contours produced by this mixture are non-elliptical, however are faithfully reproduced by the calculated pattern and showed the computed elongation index for red blood cells at nine different values of applied shear stress using two different RBC samples (Fig. 15). One sample contained erythrocytes from normal blood, while the other contained 70% normal cells mixed with 30% rigid cells.

The EI values computed using global fits are shown, as well as the EI values obtained using the LORCA's elementary ellipse-fitting routine. For normal RBC samples the global fits and the LORCA analysis produce reassuringly identical results. For the normal-plus-rigid cell population the global fits still yield the correct EI values for the normal sub-population of cells present in the sample. Note, however, that the EI curve obtained using the LORCA's ellipse fitting routine is displaced downward due to the presence of the rigid cells.

5.3. Concentration of rigid cells in a mixture with normal cells

Series of blood samples were prepared and analyzed containing different fractions of rigid cells varying between 0 and 50%. The fitting parameters (q1) and (q4) reveal the concentrations of normal and rigid cells in each sample, and the ratio q4/(q1 + q4) yields the percentage of rigid cells in each sample. A correlation between the computed fraction of rigid cells determined by the global fits and the prepared fraction of rigid cells in that sample is found (Fig. 16). At each prepared concentration, two sets of data were obtained using different apertures on the LORCA's video camera; the reproducibility of the computed results can be seen on the figure.

Although the slope of the straight-line fit is gratifyingly near unity, the intercept is not zero due to spillover of the un-diffracted portion of the laser beam as well as scattering from other objects. Such scattering is essentially the same for all shear stresses, and so mimics the diffraction pattern produced by the rigid cells. If necessary, the intercept can be brought closer to zero by excluding a larger central region of the intensity pattern from the least-square fit. The data here yield a low-stress diameter for the normal red blood cells of 8 μm, which is in agreement with the literature [7].

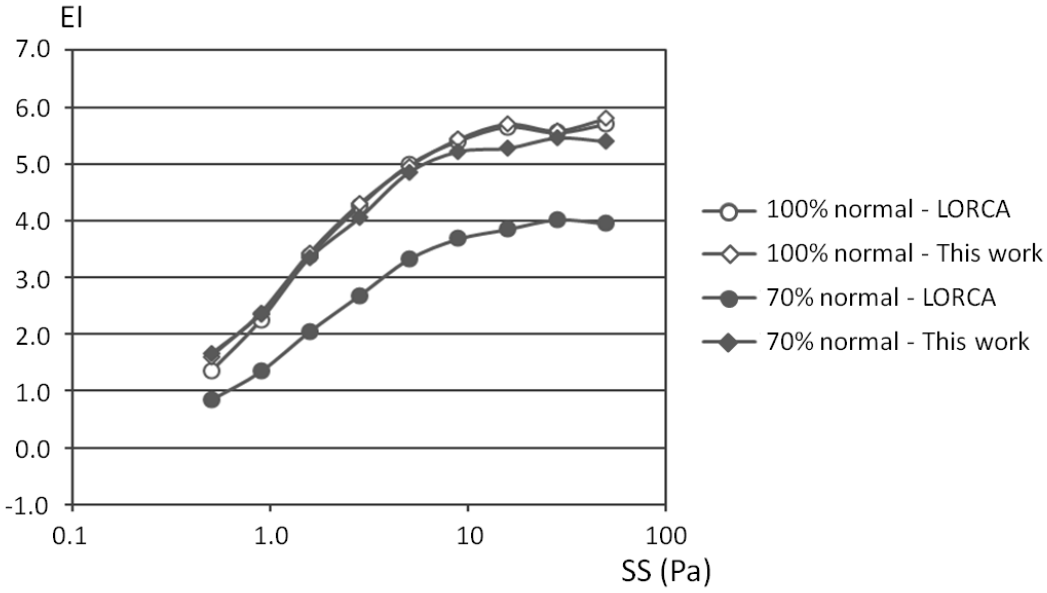

Fig. 15. *Computed Elongation Index (EI) using the LORCA's software and using the techniques presented herein. Values of two blood samples are presented here: (1) 100% normal cells shown with unfilled (open) markers. For these cells our global fits and the LORCA's ellipse-fitting routine give essentially identical EI values. (2) Mixture of 70% normal cells / 30% rigid cells. For the mixed cells, our global fits provide the correct EI of the normal cells in spite of the presence of the rigid cells. In contrast, the LORCA's ellipse-fitting routine gives EI values that are markedly reduced.*

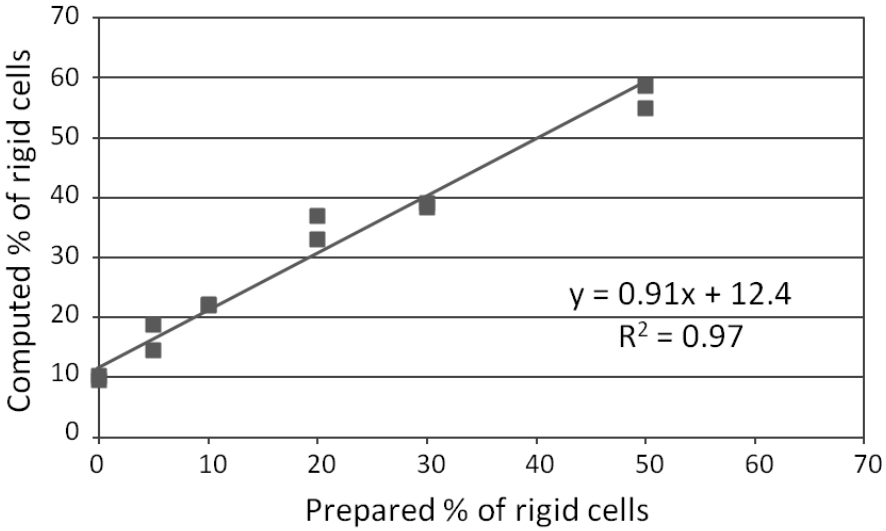

Fig. 16. *Computed fraction of rigid cells vs. the prepared fraction of rigid cells for each sample. A straight line fits to the data. The intercept of the line is not zero due to additional scattering of laser light from sources other than the rigid cells deliberately added to the sample.*

5.4. Future analysis of sickled blood

In sickled blood, it is expected that there is a continuous distribution in red blood cell deformability, while in this experiment only two cell populations (i.e., deformable and rigid) were used. Several studies have presented that the knowledge of cell deformability has clinical value. The number of circulating irreversibly sickled cells has been confirmed to be strongly correlated with the extent of hemolysis, but this correlation has not been seen in connection with the clinical severity of the disease. Furthermore, the number of rigid cells can provide information about the efficacy of therapy and may have predictive value for estimating the probability of a painful sickle crisis [12, 125].

Nevertheless, our preliminary experiments with sickled blood show that our global curve-fitting technique can accurately extract the elongation index-shear stress behavior of the normally-deforming cells in the sample. It would be also desired if this technique could estimate the percentage of rigid or barely deformable sickled cells in the blood sample.

6. Conclusion

Using a combination of Bessel functions and anomalous scattering functions to simultaneously fit ektacytometry data for multiple shear stresses can reveal the elongation index of erythrocytes over the entire range of shear stresses, even in the presence of rigid, non-deformable cells (Fig. 15).

In addition, this global fitting technique can yield the concentration of non-deformable cells in the sample (Fig. 16).

It is thus suggested that this technique will be useful in determining the curve of elongation index versus shear stress of the normal cells, as well as the concentration of rigid cells in mixed red blood cell populations as seen, for example, in sickle cell disease.

VII. Summary of new scientific results

1. Effects of red wine, alcohol-free red wine extract and ethanol

[1] Our *in vitro* measurements have demonstrated that both red wine and alcohol-free red wine extract reduce red blood cell aggregation in plasma. Red wine showed stronger inhibitory effect.

[2] Ethanol reversibly improves erythrocyte deformability and irreversibly decreases RBC aggregation.

[3] Furthermore, our experiments have revealed that alcohol-free red wine extract protects erythrocytes and preserves their deformability from oxidative stress mediated impairment.

[4] The presence of ethanol enhances the oxidative stress induced erythrocyte deformability impairment and improves the deformability of the previously damaged cells.

2. Analysis of light scattering of red blood cells in ektacytometry

[1] A new theoretical analyzer model was designed for accurately examining the diffraction patterns of ektacytometry technique.

[2] It has been proven that combination of Bessel and anomalous scattering functions reveals the elongation index of the normally-deforming red blood cells over a wide range of shear stresses in the presence of non-deformable cells.

[3] Moreover, the global curve-fitting technique yields the concentration of non-deformable cells in the blood sample.

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