Whole blood of mammalian species in the oscillating shear field: influence of erythrocyte aggregation

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Abstract. This is the rheologicalanalysis of mammalian blood of species with a high (horse), medium (man), and low (sheep) erythrocyte (RBC) aggregability by small amplitude oscillation technique. Amplitude and frequency sweep tests in linear mode were performed with blood from healthy adult volunteers, horses, and sheep in CSS-mode. Blood samples were hematocrit (HCT) adjusted (40%, 50%, 60%) and tested at 7°C, 22°C, and 37°C. Storage modulus (G´) increased with HCT and decreased with temperature in each species, but the gradient of this increase was species-specific. The lower dependency of G´ on the equine HCT value could be a benefit during physical performance when high numbers of RBCs are released from the spleen in the horse. In sheep, a HCT-threshold had to be overcome before elasticity of the blood sample could be measured, suggesting that the cohesive forces between RBCs, and between RBCs and plasma molecules must be very low. The frequencies for tests under quasi-staticcondition were in a narrow range around the physiologic heart rate of the species. In horse, time-dependent influences concurred at frequencies lower than 3 rad.s^{-1} probably due to sedimentation of RBC aggregates. In conclusion, elasticity of blood depends not only on the amount of blood cells, but also on their mechanical and functional properties.

Introduction

The rheology of blood is influencedby the quantitative and qualitative properties of blood cells, by the constitution of blood plasma, and by the adhesive and cohesive forces between blood cells and blood plasma. Compared toparticlesystems or dispersions, the viscosity of whole blood (WBV) is low, even forhighcellular fractionssuch as $40 - 45\,\%$, which is the physiological hematocrit (HCT) of man and most animal species. This is the result of the unique red blood cell (RBC) properties.

RBCs have a high surface-to-volume ratio that allowsviscous deformation in narrow vessels (1). Exposure to shear forces causes a deformation from the relaxed biconcave towards folded shapes (2-4).The RBCs return to their biconcave resting shape after disappearance of the shearing force, and even exhibit a shape memory effect (5). The RBC membrane is not static, but an active ATP-driven structure. Not only the typical shape, but also dynamic fluctuationsof the cell membrane at physiological body temperature are increased in relation to metabolic and thermal energy (6). Furthermore, RBC membranes use a tank thread motion to absorb hydrodynamic stress - a process that facilitates laminar blood flow and suspension stability within the blood vessel. The tank thread frequency starts at a certain threshold (7, 8), which is dependent on the viscosity of the suspending medium (9, 10). Another dynamic feature of RBC membranes is that they enable the cells to sustain a

rolling motion in shear flow, which avoids energetically costly deformation (8). It should be kept in mind that the volume concentration of RBCs changes dynamically throughout the body with the lowest HCT values being present in the microvasculature. As a result, the viscous resistance of blood flowing through narrow capillaries is only 20% higher than that of pure plasma (11).

RBCs form aggregates - called as Rouleaux - at low volume flow (12-15).These aggregates break up when shear rates increase at higher flows. There is remarkable diversity in RBC aggregability among the mammalian species (16). For instance, RBC aggregation is too low to be measured with routine techniques in cow, sheep, goat, mouse, and rat blood. In contrast, in horse and other equidae, the physiological RBC aggregation is as high as it would be in inflammatory disease in man (17, 18). RBC aggregation affects venous vascular resistance (19), and clinical cases associated with high RBC aggregation indicate specific diseases (20). The impact of RBC aggregability for in-vivo blood flow is discussed controversially. Although there is evidence that high RBC aggregation blunts the parabolic blood velocity profile (21), moderate RBC aggregation seems to promote blood flow (22, 23), at least in comparison to non-aggregating RBC suspensions. It is generally agreed that in-vivo blood viscosity rises when intravascular shear forces are low enough to allow RBC aggregation.By considering blood flow as Poiseuille flow (24), the lowest wall shear rates are calculated for the postcapillary venules. RBC aggregates have been seen with high-speed video microscopy in the venules after passing the postcapillary region (25). The physiological relevance of RBC aggregation lies in the phase separation phenomenon of composite fluids in narrow tubes reaching a critical diameter. Due to the axial migration of RBCs in the tube flow (26), a marginal cell-free layer (CFL) is formed, whereby the magnitude of the width of this layer (CFLW) is linked to RBC aggregability.This CFL reduces the endothelial shear stress, and subsequently, an intrinsic response mediated by vasoactive factors starts to adjust the vascular diameter (27,28). This in turn modulates the distribution of RBCs in subsequent vessels (29-31).

Rheological properties of blood are usually measured by viscosityas a function of shear rate. Methods to test properties of singular RBCs were recently summarized (32). Blood was characterized as a shear thinning viscoelastic fluid, showing different degree of thixotropy in relation to RBC aggregation. Typically, at low shear rates RBCs aggregate to clusters, while at high shear ratesRBCsare singularly suspended in blood plasma (33).

In contrast to rotational flow, small amplitude oscillating shear flow (SAOS) can be used to study the elastic property of fluids under quasi-static conditions. An application of this method to human blood has been described recently (34). Applying this principle to blood, a specific "texture"of blood will result from the forces between RBCs, and from the quality of the contact between RBCs and plasma molecules.

In the present study we tested human, equine and ovine blood atSAOS. As expected from the differences in RBC aggregability among these species, we observed that shear moduli varied, being highest in the horse. In fact, it was difficult to measure ovine blood, which likely is the result of the minute RBC aggregability of this species. It must be noted that yield stress of blood is lower than vascular wall stress under physiologic circumstances. However, intravascular shear rates are not fixed, but vary dynamically in the body. A decrease in blood flow either generalized due to blood loss or due to vascular dysfunction such as in septicaemia, or more localized due to vascular occlusion following intima hyperplasia or thromboembolic events can easily lower the wall shear rates to those shear forces that we set in our experimental protocol. Since blood is a viscoelastic fluid, storage modulus (G′) values were always below loss modulus (G′′)-values. A percolated structure in normal plasma could only be achieved at very high and thus unphysiologic HCT values. In such circumstances, blood behaved jellylike.

Material and Methods

Blood samples

Whole blood of healthy man, horse, and sheep were used for this study. 4 human volunteers (2f, 2m, age: 22-35 y), 4 *Warmblut* horses (2 mares, 2 geldings, age: 13-26 y), and 10 female *Milchschaf* sheep (age: 1.2-3.6 y) were used. 90 mL blood was withdrawn into EDTA tubes from each individual by venous puncture (man: V. radialis, horse and sheep: V. jugularis) with a 16 G needle connected to a vacutainer system. Samples were centrifuged at 2000 rpm for 5 minutes and blood plasma was separated. New samples were reconstituted out of RBC concentrate and autologous plasma to generated whole blood samples with HCT values of 40, 50, and 60%. The samples were carried in insulated bags to the laboratoryand kept in the fridge prior to their measurement. All measurements were finished within 6 hours following withdrawal.

The procedure was approved by the Ethics Committee of the Medical University Vienna (1892/2013) and the Austrian Federal Ministry of Science and Research (animal license number: GZ-1744/115-97/98).

Rheological protocol

SAOS measurements were performed using the stress controlled Physica MCR301 rheometer (Anton Paar, Graz, Austria). 3.5 mL of each blood sample was filled into the stainless steel double gap cylinder system and was analyzed at three different temperatures (7, 22, 37°C) starting with the lowest temperature. Isothermal amplitude and frequency sweep tests were performed.

Strain dependency of blood samples was measured at fixed frequency (10 rad s^{-1}) to compare the yield stresses between the species. In order to carry out the subsequent frequency tests in linear regime, some of the human and horse blood samples were tested at frequencies between 1 and 20rad s^{-1}. After a pre-shear interval (man and sheep: 30 s rotation at 300 s^{-1} followed by a 20 s interval at 1 s^{-1}; horse: 30 s oscillation at 0.01 Pa and 10 rad s^{-1}), increasing shear stresses of 0.001 – 10 Pa were applied by a logarithmic shear stress ramp. Yield points were estimated by the Rheoplus software (version 4.2, Anton Paar, Graz, Austria).

Frequency sweep tests were performed at 5-10mPathroughout the whole frequency range (20 - 1 rad s^{-1}). The blood samples were subjected to frequency sweep from high to low frequency only.

Descriptive statistic was performed by IBM®SPSS®Statistics (version 22).

Results

Amplitude sweep tests

Allowed frequencies for the frequency sweep tests were within the narrow frequency range between 3 and 15 rad s^{-1}. These frequencies correspond to the range of the physiologic heart rate of the species at rest (0.5 - 2.5 Hz).

The software easily defined the linear viscoelastic (LVE) range at 7°C in man and horse (as well as in sheep at high HCT: 60%), but became imprecise when the temperature increased. Especially at 37°C, the G´-values were sloping – although continuously decreasing. Occasionally, LVE at 37°C had to be determined based on G´´-values that always displayed a plateau until a certain shear stress was reached. As expected, experiments became more reproducible when HCT was high and temperature was low.

Basically, yield points and corresponding G´-values were very low in blood although traceable. G´-values increased with hematocrit and decreased with temperature. Data are provided in table 1 and figure 1. In blood samples at 40% HCT and 37°C,yield pointsare not provided due to increased sloping of G´, although G´´ showed a plateau value. Yield points appeared to be slightly higher in horsecompared to man. In sheep, yield points could be determined at 60% HCT only, however, the obtained values werethe highest among the three species.

Frequency sweep tests

G´-values increased with the HCT and decreased with the temperature (see table 2 and figure 2). At 40% HCT and 37°C the interquartile distance became larger indicating greater variance among the samples. There was no G´-G´´-crossover within the allowed frequencies. Sheep with the lowest RBC aggregation showed the lowest G´-values. It is interesting to note that G´ was lower in man than in horse at 40% HCT, but higher in man than in horse at 60% HCT at each temperature. At 50% HCT the median values of man and horse were nearly identical.

Table 1. Yield points(τ_y) of human, equine and ovine blood. Data are presented as median values and quartiles in parentheses

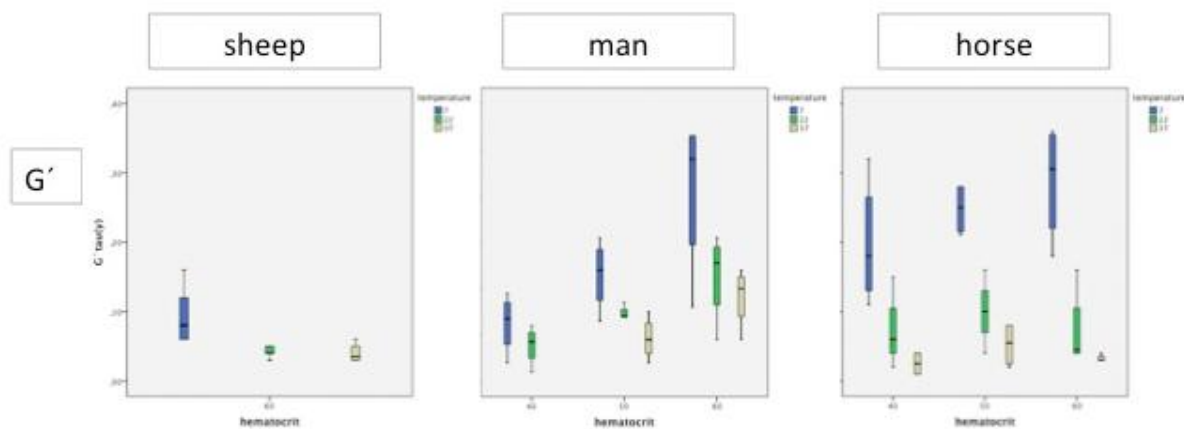| $\tau_y$at 7°C in mPa	40% HCT	50% HCT	60% HCT
Man	21(8/22)	33(13/46)	46(45/46)
Horse	33(20/86)	21(20/78)	72(13/185)
Sheep	-	-	464(348/465)
τ_y at 22°C in mPa	40% HCT	50% HCT	60% HCT
Man	3(1/8)	21(10/21)	21(12/39)
Horse	27(9/46)	20(10/30)	60(20/185)
Sheep	-	-	214(213/215)
τ_y at 37°C in mPa	40% HCT	50% HCT	60% HCT
Man	-	9(4/9)	9(4/18)
Horse	-	10(9/18)	54(4/189)
Sheep	-	-	97(31/99)

Figure 1. Amplitude sweep test: storage modulus during elastic deformationin sheep, man, and horse blood at 3 different HCT levels (40%, 50%, 60%) and 3 temperatures (blue boxes: 7°C, green boxes: 22°C, yellow boxes: 37°C). In sheep, tests could be performed at 60% HCT only.

There were only minute changes of the tanδ-value with HCT and temperature, although the loss factor decreased with the HCT and increased with the temperature in each sample. Tanδ-values at 9.38 rad s^{-1} in man were between 2.30(2.11/2.88) (measured in human blood at 60% HCT and 7°C) and 4.17(4.11/4.46) (measured in human blood at 40% HCT and 37°C).In horse tanδ-values were between 1.80(1.58/1.82) and 1.90(1.24/2.72), and in sheep tanδ-values were up to 4.56(2.98/5.38), verifying blood as viscoelastic fluid.

Table 2. Storage modulus at 9.38 rad s^{-1} in human, equine, and ovine blood samples. Data are expressed as median and quartiles in parentheses

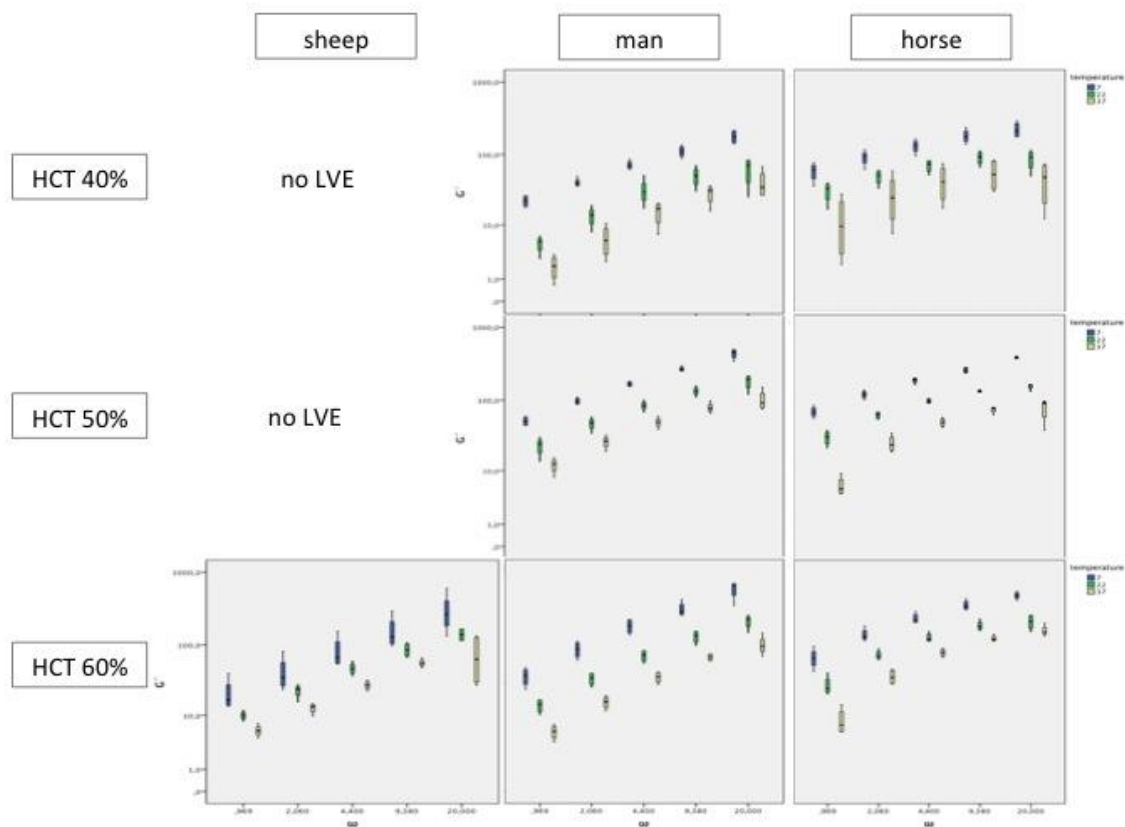| G′$_{9rad/s}$ at 7°C in mPa	40% HCT	50% HCT	60% HCT
Man	111(92/131)	262(253/289)	441(410/534)
Horse	176(145/221)	260(238/281)	340(311/415)
Sheep	-	-	130(101/258)
G′$_{9rad/s}$ at 22°C in mPa	40% HCT	50% HCT	60% HCT
Man	51(35/67)	132(116/151)	259(225/289)
Horse	92(70/109)	132(130/139)	179(161/216)
Sheep	-	-	86(69/105)
G′$_{9rad/s}$ at 37°C in mPa	40% HCT	50% HCT	60% HCT
Man	31(19/35)	77(69/92)	171(159/181)
Horse	56(31/81)	77(66/80)	119(114/135)
Sheep	-	-	55(50/63)

Figure 2. Frequency sweep test: storage modulus of sheep, man, and horse blood at 3 different HCT levels (40%, 50%, 60%) and 3 temperatures (blue boxes: 7°C, green boxes: 22°C, yellow boxes: 37°C)

Discussion

The present investigation shows that whole blood of different mammalian species can be measured by small amplitude oscillation. To study the influence of RBC properties on our protocol, we used three

species: the horse representing the species with highest RBC aggregability of all, followed by man with intermediate, and sheep with nearly immeasurable RBC aggregability (16). Using autologous plasma, the samples were adjusted to several predetermined HCT values and tested at different temperatures. By this approach, we demonstrated that the shear moduli increased with the HCT and decreased with the temperature in each species, but the gradient of this increase was species-specific.

At a frequency analog to the physiologic heart rate, G´ was higher in horse blood than in human blood at the physiological HCT (40%), but lower in horse compared toman when the HCT was raised (60%). This implicates that the gradient of the G´-increase in relation to the cellular fraction in blood was lower in the horse. This could be of physiological relevance in this species. Resting horses possess a splenic reservoir for RBCs with a HCT of about 80%. After an exercise-induced contraction, these splenic RBCs are added to the RBCs in the circulation thereby increasing HCT to maximally 65% (35). The extra number of circulating RBCs effectively increases the blood oxygen store and the oxygen carrying capacity. However, in parallel, the blood viscosity and the elasticity of the blood suspension is elevated, as well. On the other hand, the low dependency ofG´on the HCT valuecould be a benefit during exercise.

In sheep, a HCT-threshold must be overcome before elasticity ofthe blood samplewas detected.Ovine RBCs are smaller than those of man and horse (36) and have a reduced deformability if exposed to shear stress (37). Based on their minute RBC aggregability (16), RBCs are singularly suspended in the plasma volume. Flow curves show the low shear thinning of ovine blood. Our results notice that apart from the attracting forces between the RBCs, also the forces between RBCs and the surrounding plasma should be low.

Another interesting finding of the sheep blood concerns the yield point that could not be measured at HCT of 40% and 50%. However, at HCT of 60% the yield point was higher than in man and horse. The physiological HCT of ovine blood ranges between 30 and 38% (36). A release of splenic RBCs by a sympathetic stimulus is insignificantin sheep due to the diverse composition and function of the spleen. Sheep do not exhibit high HCT values regularly. But if they do so, the yield stress of blood ishigherthan in man and horse.

In conclusion, blood is a fragile suspension that shows its best stability around the resting heart rate of the particular species. At frequencies above 15 rad $s^{-1}$and below 3 rad s^{-1}, G´-values weresignificantly sloping, showed an irregular sequence, or were even absent. We assume that the weak cohesive forcesof bloodare disrupted at high frequencies, while at low frequencies, sedimentation of RBCs in the rheometer gap may result in phase separation and shear banding during the measurement. It is logical thatsuspension stability will increase with the increase of the volume fraction of cellular elements in a composite fluid. In addition, we showon our HCT standardized samples that G´-values and yield stresses are higher when RBCs have the chance to form aggregates.

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