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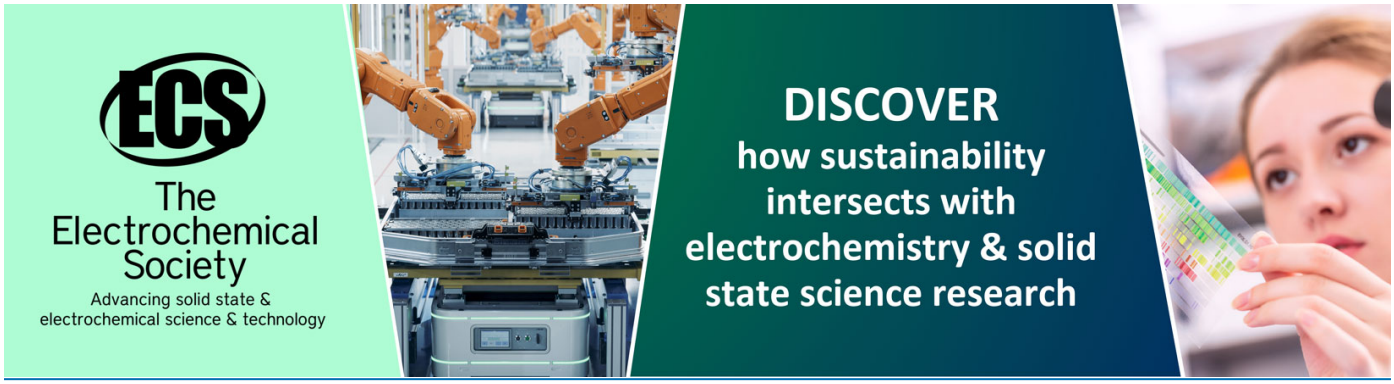

Measurement of whole blood of different mammalian species in the oscillating shear field: influence of erythrocyte aggregation

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Abstract. This is the first systematic analysis 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 (linear viscoelastic 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. Generally, 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 sheep, an HCT-threshold had to be overcome before the desired quasi-static condition of the blood sample could be achieved, suggesting that the contact between RBCs, and between RBCs and plasma molecules must be very low. The frequencies for tests under linear viscoelastic condition 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, blood is a fragile suspension that shows its best stability around the resting heart rate of the species.

1. Introduction

The rheology of blood is influenced by the quantitative and qualitative properties of blood cells, by the constitution of blood plasma, and by the attracting forces between blood cells and blood plasma. Compared to particle systems or dispersions, the viscosity of whole blood (WBV) is low, even for high cellular fraction such as 40- 45%, which is the physiological hematocrit (HCT) of man. This is the result of specific red blood cell (RBC) properties.

RBCs have a high surface-to-volume ratio that allows viscous 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 fluctuations of 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. Tank thread frequency

starts at a certain shear rate 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 easily 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 rather seem to promote blood flow (22, 23), at least in comparison to non-aggregating RBC suspensions. It is generally agreed that in-vivo 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, RBC aggregates and clusters, while at high shear rates singularly suspended RBCs contribute to plasma enhancement (33).It must be noted that low shear viscosity reflects the specific texture of blood at low shear which is given by the sum of all attracting forces in the respective sample, rather than showing RBC aggregation alone.

In contrast to rotational flow, small amplitude oscillating flow (SAOS) can be used to study fluids under quasi-static conditions. An application of this method to human blood has been described recently (34). The rheological material characterization by SAOS tests the association of components within a sample and includes the forces between the filler materials plus the forces between filler and matrix. 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 at small amplitude oscillatory shear flow. As expected from the differences in RBC aggregability among these species, we observed that shear moduli varied between these three species, being highest in the horse at physiological HCT. In fact, it was difficult to measure ovine shear moduli, which likely is the result of the minute RBC aggregability of this species. It is worth noting that SAOS had to be performed at slightly lower shear stresses than those present in the vessels under physiologic circumstances. However, vascular 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 unphysiologic HCT (higher than 75%). In this circumstance, a jellylike texture was the result.

2. Material and Methods

2.1. 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 (2mares, 2geldings, 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 10 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 30, 40, 50, and 60%. The samples were carried in insulated bags to the laboratory and kept in the fridge prior to their measurement. All measurements were finished within 8 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).

2.2. Rheological protocol

SAOS measurements were performed using the stress controlled PhysicaMCR301 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 0.01 Pa throughout 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).

3. Results

3.1. 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 LVE-range could be easily defined by the software 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 G´-values at LVE 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 points are not provided due to increased sloping of G´, although G´´ showed a plateau value. Yield points appeared to be slightly higher in horse compared to

man. In sheep, yield points could be determined at 60% HCT only, however, the obtained values were the highest among the three species.

3.2. 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 point of human, equine and ovine blood. Data are presented as median values and quartiles in parentheses

$\tau_y$at 7°C in Pa	**40% HCT**	**50% HCT**	**60% HCT**
Man	0.021(0.008/0.022)	0.033(0.0127/0.046)	0.046(0.045/0.046)
Horse	0.033(0.020/0.086)	0.021(0.020/0.078)	0.072(0.013/0.185)
Sheep	-	-	0.464(0.348/0.465)
$\tau_y$at 22°C in Pa	40% HCT	50% HCT	60% HCT
Man	0.003(0.001/0.008)	0.021(0.010/0.021)	0.021(0.012/0.039)
Horse	0.027(0.009/0.046)	0.020(0.010/0)	0.060(0.020/0.185)
Sheep	-	-	0.214(0.213/0.215)
$\tau_y$at 37°C in Pa	40% HCT	50% HCT	60% HCT
Man	-	0.009(0.004/0.009)	0.009(0.004/0.018)
Horse	-	0.010(0.009/0.018)	0.054(0.004/0.189)
Sheep	-	-	0.097(0.031/0.099)

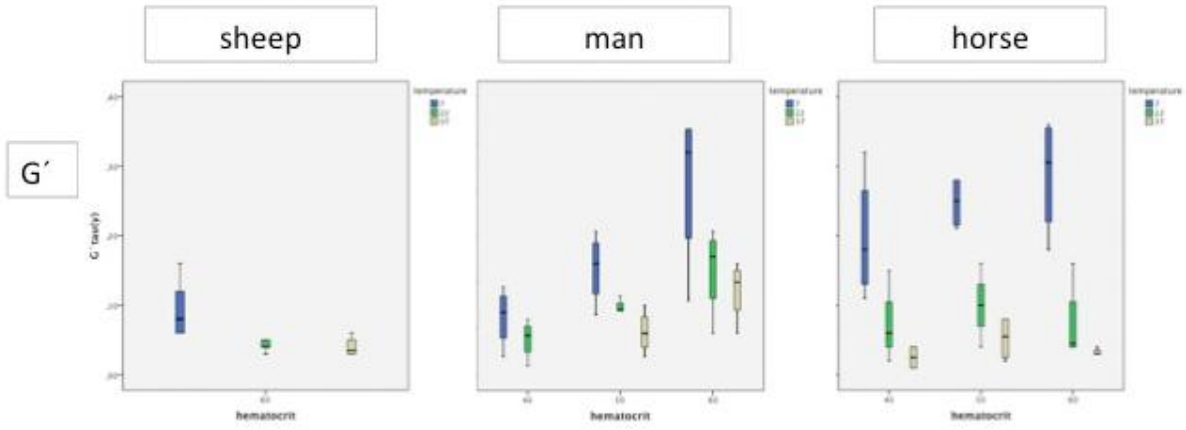

Figure 1. Amplitude sweep test: storage modulus at LVE in sheep, man, and horse blood at 3 different HCT levels (40%, 50%, 60%) and 3 temperatures (blue boxes: 7°C, green boxes: 22°C, yellow boxes: 37°C). In sheep, tests could be performed at 60% HCT only.

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} ranged 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), between 1.80 (1.58/1.82) and 1.90

(1.24/2.72) in horse, and up to 4.56 (2.98/5.38) in sheep, verifying blood as viscoelastic fluid. Data are provided in figure 3.

Table 2. G´ 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 Pa	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 Pa	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 Pa	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)

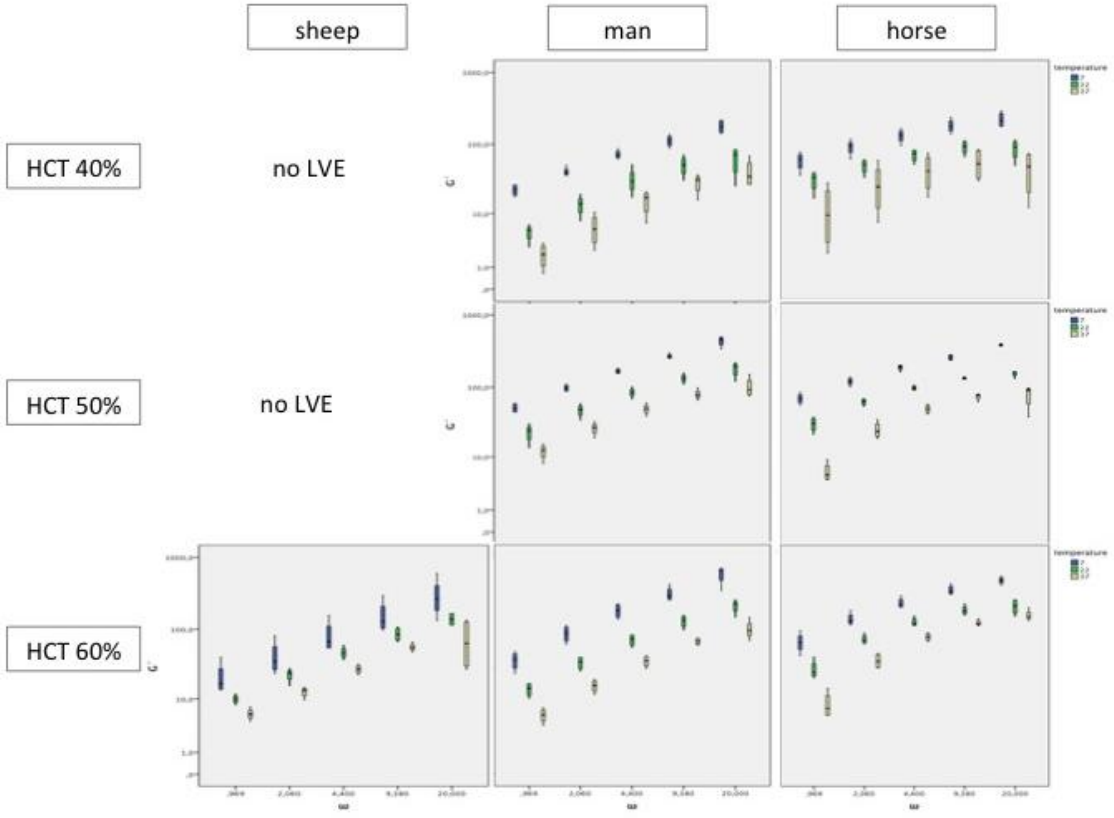

Figure 2. Frequency sweep test: storage modulus of sheep, man, and horse blood at 3 different HCT levels (40%, 50%, 60%) and 3 temperatures (blue boxes: 7°C, green boxes: 22°C, yellow boxes: 37°C).

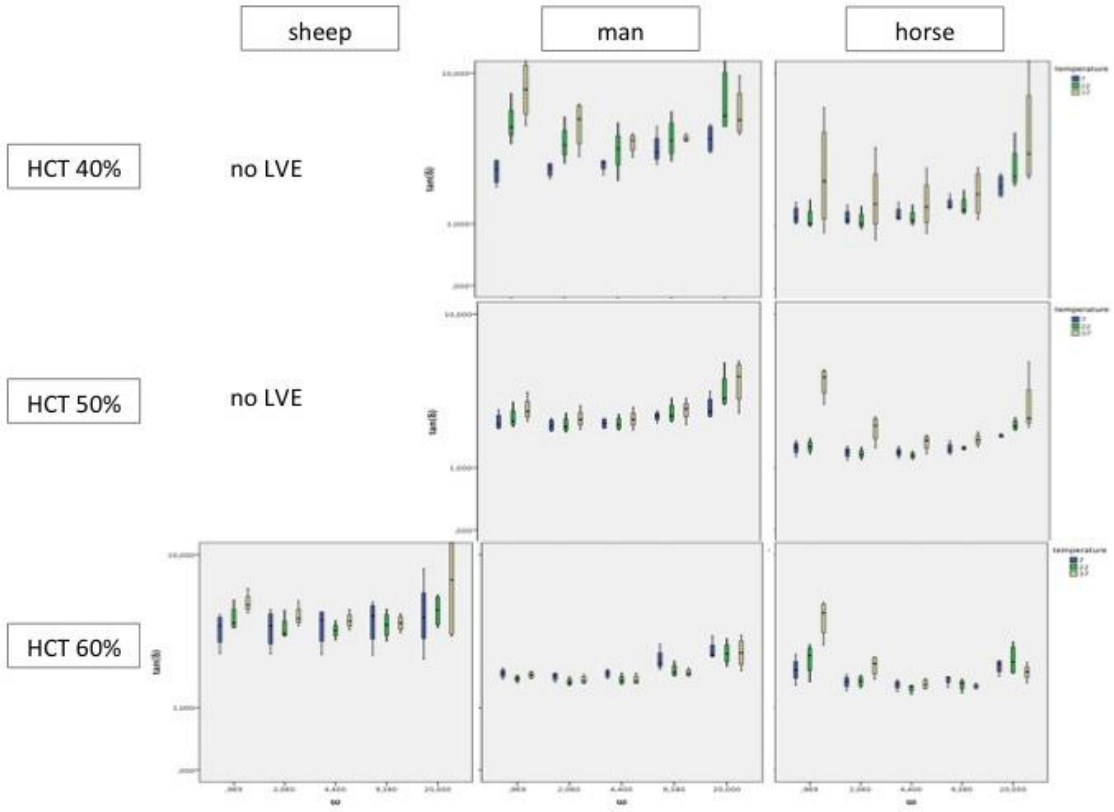

Figure 3. Frequency sweep test: loss factor (tanδ) of sheep, man, and horse blood at 3 different HCT levels (40%, 50%, 60%) and 3 temperatures (blue boxes: 7°C, green boxes: 22°C, yellow boxes: 37°C).

4. Discussion

In the present investigation we showed that whole blood 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 to man 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 about80%. 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 elastic proportion of the blood suspension is elevated, as well. On the other hand, the low dependency of G´ on the HCT value could be a clear benefit during exercise.

In sheep, an HCT-threshold must be overcome before the desired quasi-static condition of the blood sample is achieved. 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 thus singularly suspended in the plasma volume. Flow curves show the low shear thinning of ovine

blood. Our SAOS results notice that apart from the attracting forces between the RBCs, also the forces between RBCs and the surrounding plasma must be very 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 is 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 insignificant in 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 structural strength of blood that is reflected by the yield point seems to be higher than 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 are significantly sloping, show an irregular sequence, or are even absent. We assume that the weak structure of the bulk is disrupted at high frequencies, while at low frequencies, sedimentation of RBCs in the rheometer gap resulted in phase separation during the measurement. It is logical that the suspension stability increased with the increase of the volume fraction of cellular elements. Concerning the relevance of RBC aggregability for the suspension stability, it was shown that G´-values are higher when RBCs had the chance to form aggregates, at least at physiological circumstances.

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