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Pig and Cow Blood During Cold Storage in CPDA-1 Solution: Hematology and Fluid Behavior

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Abstract: Nature equipped red blood cells (RBCs) with diverse mechanical properties, which makes it possible to examine blood with different RBC properties (size, shape, aggregability, deformability). We investigated whether the shelf life of cow blood (stiff RBCs, low aggregability) is longer compared with pig blood (deformability/aggregability comparable to human) due to a delay in RBC clustering and decomposition. Blood was drawn from conscious pigs and cows in their familiar environment to reduce stress and stored 30 days at +7 °C. RBCs remained intact in cow samples whereas pig samples became hemolytic after day 20. White blood cells and platelets decreased with similar percentages in both species. Hematocrit (HCT) decreased due to RBC shrinking in bovine samples and due to RBC decay in porcine samples. Blood viscosity increased in both species although HCT decreased. In porcine samples, shear thinning decreased progressively, indicating a gradual loss of sample cohesion with storage. Yield stress and storage modulus decreased with hemolysis. In HCT-native cow samples, shear thinning, yield stress, and storage modulus showed high intraindividual variability, but the mean values did not change over the time course. In HCT-adjusted (38%) cow samples, solidification occurred after day 7, followed by a reduction in cohesion and shear thinning until the end of storage.

Keywords: cow blood ageing; pig blood ageing; rheology; hematology; shelf life

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1. Introduction

Animal blood is used in biomedical research if practical or ethical considerations do not allow the use of human blood and if artificial solutions are not available or do not fulfill the desired demands. In mock circulations or device testing apparatus, large blood volumes are needed, and only large animals can serve as donors [1], among which are ruminants and pigs. Due to the propensity of equine red blood cells (RBCs) to spiculate and their pronounced aggregability [2], horse blood is unsuitable for most applications. Animal blood can generally be viewed as an experimental model system that offers the possibility of studying blood suspensions containing erythrocytes (RBCs) with various intrinsic and extrinsic properties. The benefit is the presence of native suspensions in which the natural cohesion between the components is unaltered. Attempts to chemically stiffen RBCs by aldehyde incubation, or to modify their aggregation by adding dextran, generate unphysiological blood.

We investigated the fate of two blood suspensions, which differ in their bulk behavior [3]. We investigated whether a suspension containing stiff, non-aggregating RBCs might have a longer shelf life during refrigerated storage than a suspension containing deformable RBCs that aggregate into cell clusters. The advantage of stiff RBCs could be

their membrane properties that make them less susceptible to blebbing and vesiculation. This hypothesis is supported by the recent work of Kennedy and coworkers, who showed that bovine RBCs are less prone to shape changes after exposure to oxidative stress [4]. The advantage of non-aggregating RBCs could be the lower tendency to form clusters and debris [5], thus reducing the phase separation processes when aged blood is sheared in mock circulation. These assumptions suggest ruminant blood, with its stiff and less aggregating RBCs [3], as a better choice for applications in biomedical research or in the forensic discipline of bloodstain pattern analysis [6,7]. However, pig blood is more comparable to human standards [8] and pigs, rather than cows or sheep, are used as a trauma model in biomedical research when blood products are involved [9,10]. It is also the (genetically modified) pig that is considered as a donor for xenotransfusion [11], which puts the pig more in focus. Another advantage of pig blood is the HCT value, which is comparable to that of humans, whereas the HCT of ruminants is much lower, affecting the flow resistance in circuits.

In the following, we compare the changes in the suspension stability of pig and cow blood during 30 days of cold storage. Our study design was observational, and combined rheology and hematology. The time point at which cohesion starts to become significantly altered is identified as the shelf life.

2. Materials and Methods

2.1. Blood Samples

Blood was drawn from eleven pigs (age: 4.5 months, 8 females, 3 castrated males) and eleven cows (age: 5.5 $\pm$ 2.4 years), by puncturing the V. cava cranialis (pigs) and the V. jugularis (cows). Blood was drawn into 50 mL syringes that were prepared as follows. Citrate-Phosphate-Dextrose-Adenine-1 (CPDA-1) was extracted from standard CPDA-1 blood bags (Fresenius Kabi, Bad Homburg vor der Höhe, Germany) and inserted into sterile perfusor syringes (Braun, Melsungen, Germany; 7 mL of CPDA-1 + 43 mL of blood, resulting in a 1:7 dilution of blood). The syringes were connected to short sterile IV lines attached to sterile needles. All air was removed from these systems prior to punctuation of the vein and blood was drawn to the 50 mL mark. The skin of the animals was cleaned by using a skin disinfectant (Sterillium®, Bode Chemie, Hamburg, Germany) prior to punctuation. Blood samples were used as drawn in eleven pigs and in six cows. Due to the physiological species-specific differences of RBC size and count, the HCT is different between the blood types. To compare samples of equal HCT, blood of five additional cows was adjusted to the porcine HCT value (38.4 $\pm$ 1 %) by centrifugation (1200$\times$ *g*) and resuspension of RBC concentrate and plasma. HCT-native samples were stored at 4 °C for 30 days and analysed every second (pig) or third (cow) day. HCT-adjusted bovine blood was stored at 4 °C for 28 days and analysed every seventh day. Prior to each analysis, the syringes were placed in an overhead agitator (REAX2, Heidolph, Schwabach, Germany) at the lowest available speed for a minimum of ten minutes to allow tempering and resuspension of blood cells. For performing the porcine blood smears, blood was taken from three pigs of another cohort but same race (Large White $\times$ Landrace) present in the same institution at a later occasion, to be stored in the fridge for 30 days.

Withdrawal of blood was approved by the institutional ethics and animal welfare committee and the national authority according to §§ 26ff. of Animal Experiments Act, Tierversuchsgesetz 2012—TVG 2012, reference number BMBWF-68.205/0092-V/3b/2019 (cows) and BMWF-66.009/0372-WF/V/3b/2014, resp. BMWFW-68.205/0188-WF/V/3b/2015 (pigs).

2.2. Hemograms and Free Hemoglobin

Hematology was performed using an ADVIA 2120i system (Siemens, Erlangen, Germany) that applies photometry, flow cytometry, and impedance measurement. To observe the onset of hemolysis, aliquots of the blood samples were centrifuged using a Haematokrit 2010 centrifuge (Hettich, Tuttlingen, Germany). A change in the colour of blood plasma in the glass capillaries was assessed visually. RBC shape was assessed microscopically in bright field at days 0 and 30 (Olympus IMT-2 mounted to a Nikon DS-Fi1 camera/DS-U3 digital sight tool, Tokio, Japan) in all cow samples and in three pig samples. Plasma-free hemoglobin (fHb) was measured in pig samples with a colorimetric method using a Cobas c 311 analyser (Roche, Basel, Switzerland).

2.3. Rheometry

Physica MCR 301 and 302 rheometers (Anton Paar, Graz, Austria) equipped with a Peltier-controlled steel double gap cylinder (pigs: internal gap: 0.417 mm, external gap: 0.462 mm, cup length: 42 mm) or with a cone-plate shear cell (cows: diameter: 50 mm, angle: 0.992°, truncation: 0.100 mm) were used. The cone-plate cell was mounted to the rheometer within with a tempered hood equipped with an evaporation blocker to avoid sample drying. Blood exactly filled the whole gap; there was no asymmetric filling or overfilling. Before blood was filled in the gap, the test surfaces were cleaned by a detergent followed by extensive rinsing with distilled water and 98% ethanol. Thereafter, the surfaces were dried with a fan and mounted to the rheometer so that they could be perfectly tempered before the blood was filled in. Tests were performed at 22 °C.

2.3.1. Tests in Simple Shear Flow

To obtain shear viscosity (η), we created shear strain-controlled flow curves (shear rates 1000–10 s^{-1} on a logarithmic shear rate ramp; 11 data points). The software calculates viscosity from the shear stress versus shear strain relationship ($\eta\ [Pa \times s] = \tau\ [Pa]/\dot{\gamma}\ [s^{-1}]$).

2.3.2. Tests in Oscillatory Shear Flow

New portions of blood were exposed to increasing shear stress amplitudes at constant 1 Hz frequency (1–1000 mPa, logarithmic ramp), as well as to increasing frequencies (0.5–5 Hz in cows; 0.1–10 Hz in pigs; logarithmic ramp) at dynamically (sinusoidally) changing shear stresses with maximal amplitude of 10 mPa. The amplitude sweep tests served to identify the linear viscoelastic range (LVER) and the yield points of the suspensions. The LVER was ≥10 mPa at start due to the frequency dependence, which is why this amplitude was selected in the frequency sweep tests in both species. From the frequency spectrum, the shear moduli (G′, G″) at 1 Hz (pig) or 0.8 Hz (cow) were extracted and the loss factor was calculated.

Tests in the oscillating shear field were used to obtain the shear moduli of the samples. The storage (same as elastic, G′) and loss (same as viscous, G″) moduli were calculated out of the stress–strain relationship during the sinusoidal change in time and amplitude ($G^* = \tau(\omega,A)/(\omega,A)$), by using the phase shift angle (δ). The phase shift angle identifies the lag phase between the applied shear stress and the resulting shear strain. It decreases the more a material changes from fluidic to elastic; G′ equals G* cosδ; G″ equals G* sinδ. Both values can be combined in the loss factor ($\tan\delta = G''/G'$). The yield stress was extracted manually from the G′-curve in the amplitude sweep tests by using the method described in [12].

2.4. Data Processing and Statistics

The following software programs were used for data handling, analysis, and illustration: RheoCompass (version 1.22; Anton Paar, Graz, Austria), Excel (version 2016; Microsoft,

Redmont, Washington, DC, USA), GraphPad Prism (version 6; GraphPad, La Jolla, CA, USA), and NIS-Elements (version D 4. 13.5; Nikon, Tokio, Japan). For comparative reasons, hematological values and blood viscosity are presented in Figures 1 and 2 as relative changes to baseline—except for HCT due to the high species-specific difference of HCT between cow and pig. Rheological parameters obtained in the dynamic shear field are presented as absolute values. Data are displayed as mean ± SD. The Wilcoxon matched pairs signed rank test was used to analyse if values differ at storage day 30 compared with storage day 0 ($\alpha = 0.05$).

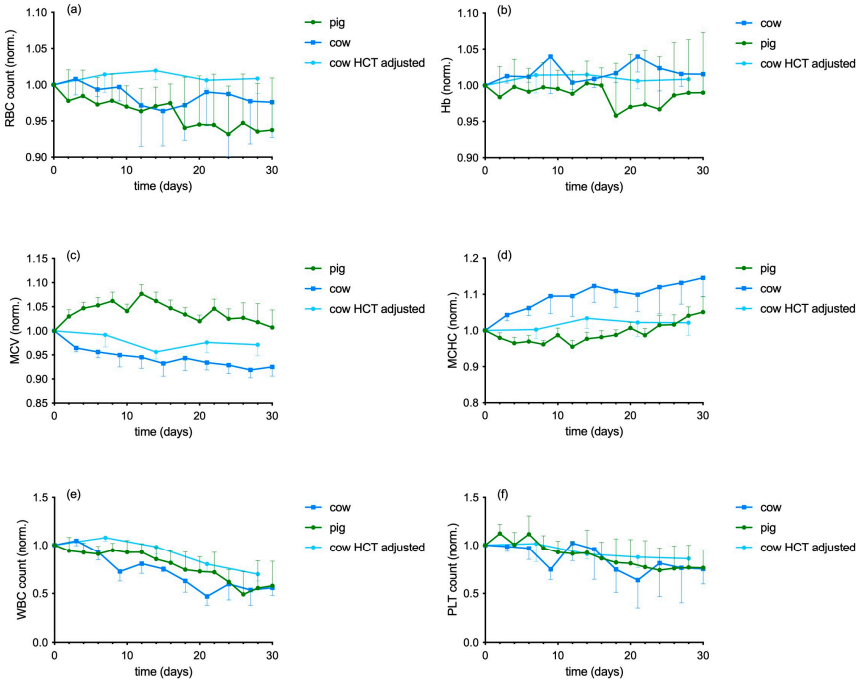

Figure 1. Change in the hemograms of porcine (n = 11) and bovine (n = 6) whole blood samples with storage time. (**a**,**b**): RBC count and Hb; (**c**,**d**): MCV and MCHC; (**e**,**f**) WBC and PLT count. Data present mean ± standard deviation of changes relative to baseline.

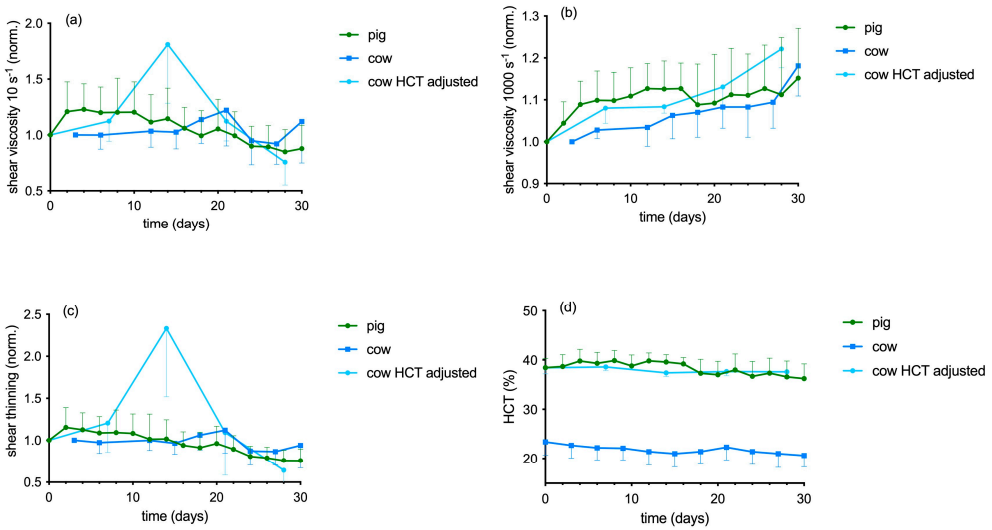

Figure 2. Change in the rheological behavior and HCT of porcine (n = 11) and bovine (n = 6) whole blood samples with storage time. Rheological data present mean ± standard deviation of changes relative to baseline. (**a**,**b**): Shear viscosity at low and high shear rates. High shear rate viscosity increased continuously in all samples, but the low shear rate viscosity value peaked at day 14 in HCT-adjusted bovine samples, representing blood thickening; (**c**): shear thinning (η_{10}/η_{1000}) decreased in porcine and HCT-adjusted bovine samples towards the end of the observation period as an indicator of a deteriorating suspension but not in HCT-native bovine samples. The significant thickening of HCT-adjusted bovine samples is also reflected in the rise of shear thinning on day 14. Due to the species-specific difference, the HCT is displayed as absolute values. Technical problems prevented rheometry of HCT-native bovine samples on day 0. (**d**): Change in HCT in the form of absolute values.

3. Results

3.1. Hemograms and Hemolysis

Bovine RBCs remained intact in contrast to porcine RBCs. In the blood smears of aged pig blood, ghosts and debris were found, whereas in cow blood slightly smaller round RBCs were present and there was no debris (Figure 3). In addition, the RBC count was better preserved in cow blood samples than in pig blood samples. WBC counts were species-specifically higher in pig than in cow, but in both species, the WBC count of HCT-native samples decreased by ≈43%. The PLT counts of HCT-native samples decreased by 25% in both blood types (Table 1, changes relative to baseline are presented in Figure 2). A smaller decrease in WBC and PLT counts was observed in HCT-adjusted cow samples.

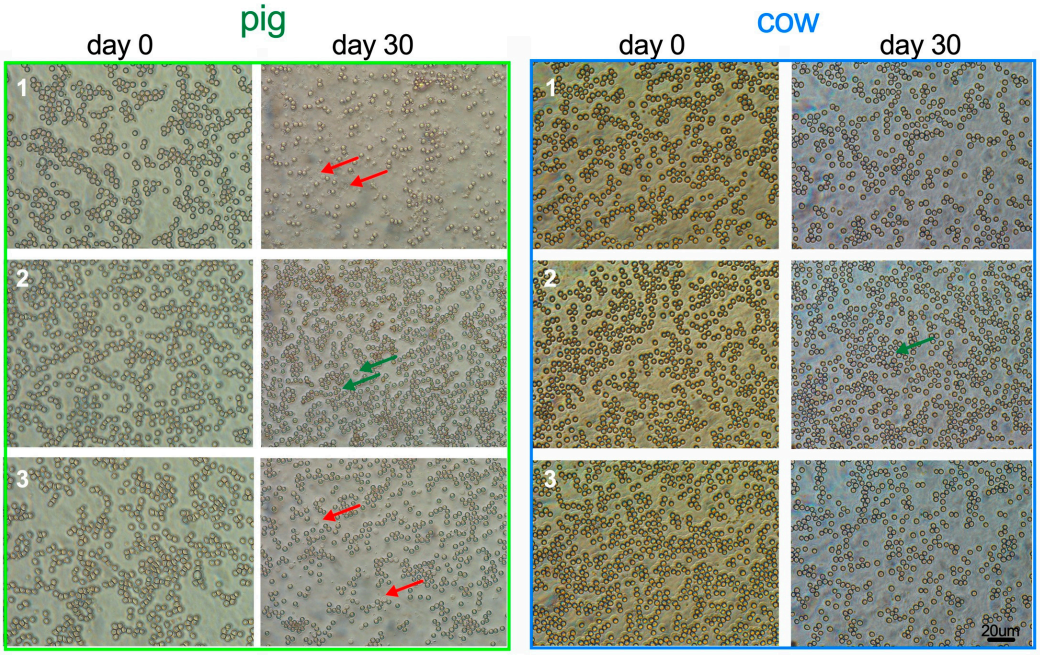

Figure 3. Blood smears of porcine and three selected bovine blood samples at the beginning and the end of storage. After 30 days of storage: pig 1: ghosts (red arrows) and cell debris; pig 2: crenated cells (green arrows); pig 3: ghosts. After 30 days of storage: cow 1 and 3: regular round shapes; cow 2: crenated cells. Scale bar: 20 µm.

Table 1. Cell counts in bovine and porcine samples at start and end of refrigerated storage. Asterisks mark the significance level (* $p = 0.05$; ** $p = 0.01$).

	Day 0 Cow	Day 30 Cow	Cow	Day 0 Pig	Day 30 Pig	Pig
RBC count (T/L)	4.96 ± 0.7	4.75 ± 0.7	−4.2%	7.33 ± 0.4	6.86 ± 0.4 **	−6.4%
WBC count (G/L)	6.01 ± 1.1	3.4 ± 1.0 *	−43%	15.5 ± 3.3	8.3 ± 3.9 **	−44%
PLT count (G/L)	266 ± 77	198 ± 67	−25%	262 ± 43	200 ± 63 **	−25%
WBC count (G/L) HCT-adjusted	9.61 ± 1.89	6.59 ± 0.65	−30%		-	
PLT count (G/L) HCT-adjusted	443 ± 83	374 ± 38	−14%		-	

Porcine RBCs swelled during the first 14 days of storage and returned to their initial volume at the end of storage time (n.s.), whereas bovine RBCs shrank continuously without initial swelling. Thus, the bovine RBCs became successively dense with storage time, indicated by the continuous increase in MCHC that reached a maximum of +12% on day 32 in HCT-native samples. In HCT-adjusted samples from a different group of cows, RBC count and MCHC were better maintained during storage, and the cells shrank less. In pig blood, the MCHC was maintained up to day 22 of storage due to the initial cell swelling but increased thereafter as well (all $p < 0.05$).

Hemolysis occurred in all pig blood samples and became severe from day 22: mean plasma free hemoglobin concentration was of over 200 g dL^{-1} on day 30. In contrast, the cow plasma did not show signs of hemolysis during the whole storage period, except in one case. In this cow sample, the plasma was severely reddish on day 9. The analyser did not identify a reduction in RBC count on that day (4.66 vs. 4.54 T/L at start of storage), suggesting that membrane damage had just started. Three days later, the RBC count and the HCT were reduced and remained on a lower level throughout. The MCV of the remaining RBCs in this sample was fairly constant until day 18 of storage, when this value decreased, as in the other samples.

The combined changes of cell count and cell volume affect the HCT, which has a major influence on the rheology of blood. HCT decreased with storage time in both species (Figure 2d), but with different quantitative and temporal characteristics. The initial RBC swelling prevented the HCT decrease in pig blood during the first two weeks, although the RBC count decreased right from the beginning. A progressive hemolysis started on day 20, which reduced the HCT significantly in the second half of the observation period. In contrast, the HCT decreased right from the beginning in cow blood, as no cell swelling occurred (both species $p < 0.05$). To note, due to the use of native blood and the species-specific difference in HCT, the bovine HCT value was always less than half that of the pig samples. Since the representation of changes relative to the baseline are misleading if the initial values vary strongly, the change in HCT with storage duration is shown in the form of absolute values in Figure 2d. Low shear viscosity of HCT-adjusted bovine samples peaked on day 14, which led to significant rise in shear thinning, indicating the thickening of the samples.

3.2. Rheology

3.2.1. Tests at Simple Shear Flow (Blood Viscosity)

In both species, the high shear rate (1000 s^{-1}) viscosity increased continuously (cow HCT-native $p < 0.05$; cow HCT-adjusted $p < 0.01$; pig $p < 0.01$; Figure 2b), although at the same time, the HCT decreased.

In both species, the low shear rate (10 s^{-1}) viscosity was not different at storage day 30 compared with day 0. However, the timely course of this viscosity value varied between the species. In pig, it showed a relationship to HCT. HCT increased during the first ten days due to RBC swelling and so did the low shear viscosity. HCT decreased thereafter due to the RBC decay to values a little below the baseline, and the low shear viscosity followed this course. The minute decrease in low shear rate viscosity along with the significant increase in high shear rate viscosity resulted in a continuous reduction in shear thinning (η_{10}/η_{1000}) during the storage of pig blood ($p < 0.001$). In contrast, in HCT-native cow blood, no change in shear thinning was present (n.s.; Figure 2c). This is because low shear viscosity mostly varied around the baseline value up to the 24th day of storage. Thereafter, in half of the samples, the value increased, whereas in the other half of the samples—including the haemolytic sample—it decreased, giving rise to a mean value not different from the baseline. The development of low shear rate viscosity in HCT-native cow blood did not follow the course of HCT and appeared unpredictable. In HCT-adjusted cow blood, however, a significant rise in viscosity occurred at storage day 14, followed by a decrease to baseline values at day 28. This points to a temporary thickening of the suspension while maintaining cohesion.

3.2.2. Tests at Oscillating Shear Flow (Shear Moduli)

Amplitude Sweep Tests

The LVER was $\geq$10 mPa in all pig and cow samples at the start of storage. However, during storage, it became increasingly difficult to discern a truly linear behavior of the samples as the G′ curves at low shear deformation plateaued less and less as the samples aged. This means that it was progressively difficult to assess the yield stress value directly from the G′-curve. In many cases, it was necessary to apply a quadratic regression to the G′-values and to read the yield stress from the regression curve. Apart from the gradual loss of the quasi-static behavior within the observable shear stress range, there was also an intraindividual variability in the yield stress values in the samples during the time course (see Figure 4c,d). This variability of yield stress values was much higher in cow samples than in pig samples. In cow samples, it did not allow for the detection of a trend

towards more solidifying or more fluidifying with the progression of storage (Figure 4a,e). In contrast, the yield stress of the pig samples decreased continuously during the time course, albeit with high error bars (Figure 4b). At storage day 30, the yield stress was three times lower than at the start (Figure 4f, $p < 0.01$). In addition to the yield stress, we also drew the degree of solid-like property from the amplitude sweep tests. Due to the fact that a true G′-plateau was difficult to detect as the ageing proceeded, we pooled the first three G′-values of the test (values between 1 and 5 mPa shear stress and constant 1 Hz) and calculated the mean value out of it. In the cow samples, these mean G′ values were 56 ± 45 mPa on day 3 and 55 ± 37 mPa on day 30 (compare with Figure 4e; n.s.). In the pig samples, the mean G′-moduli decreased with storage duration (115 ± 110 at day 0 and 48 ± 35 mPa on day 30; $p < 0.01$), supporting the sample fluidification with storage time (compare with Figure 4f).

Frequency Sweep Tests

The difficulty of aging HCT-native cow samples to remain in a quasi-static state is also reflected in the timely change in the loss factor. This parameter, which combines both shear moduli to one value, showed high intraindividual variability (Figure 5c), resulting in no change in mean values during the time course, except once due to the exorbitantly high loss factor value (reflecting high fluidity) of cow E after 24 days of storage (Figure 5e). This is supported by the frequency spectra of cow blood obtained before and after storage (Figure 5a); the curves do not indicate any change in suspension behavior. In contrast, the loss factor increased in all pig samples (Figure 5d) after a lag of 3 weeks. This reduction in sample cohesion after the 20th day of storage ($p < 0.05$) occurred in parallel with the onset of hemolysis. The frequency spectra of pig samples before and after storage thus indicated the material deterioration: G'_{1Hz} decreased from 69 ± 55 mPa at day 0 to 23 ± 11 mPa at day 30 ($p < 0.001$); G''_{1Hz} decreased from 91 ±26 mPa at day 0 to 68 ± 17 mPa on day 30 ($p < 0.05$; Figure 5b).

3.2.3. Summary of Similarities and Differences Between Pig and Cow Blood During Ageing

RBCs remained intact in cow samples whereas there was significant hemolysis in pig samples after day 20. WBCs and PLTs decreased with similar percentages in both species. HCT decreased due to RBC shrinking in cow samples and due to RBC decay in pig samples. Blood viscosity at a high shear rate increased in both species although HCT decreased. Blood viscosity at a low shear rate was unchanged in both species if one compares the values at the start and end of storage. However, the processes during storage are not trivial. The pig samples displayed a reduction in shear thinning, indicating a gradual loss of sample cohesion with time. This conclusion is supported by the three times smaller yield stress and storage modulus at storage day 30 compared with day 0, and by the rise of loss factor in parallel with the degree of hemolysis. In HCT-native cow samples, we did not observe changes in shear thinning, yield stress, storage modulus, and loss factor with storage duration. However, the intraindividual variability of those values that are obtained in the low shear field during the several days of storage was high. The mechanical properties of HCT-native cow blood suspensions thus develop unpredictably. In contrast, increasing HCT to values comparable to those in pigs led to a predictable but at the same time pathological behavior; the samples transiently solidified before approaching the Newtonian plateau, which is the signature of suspension instability.

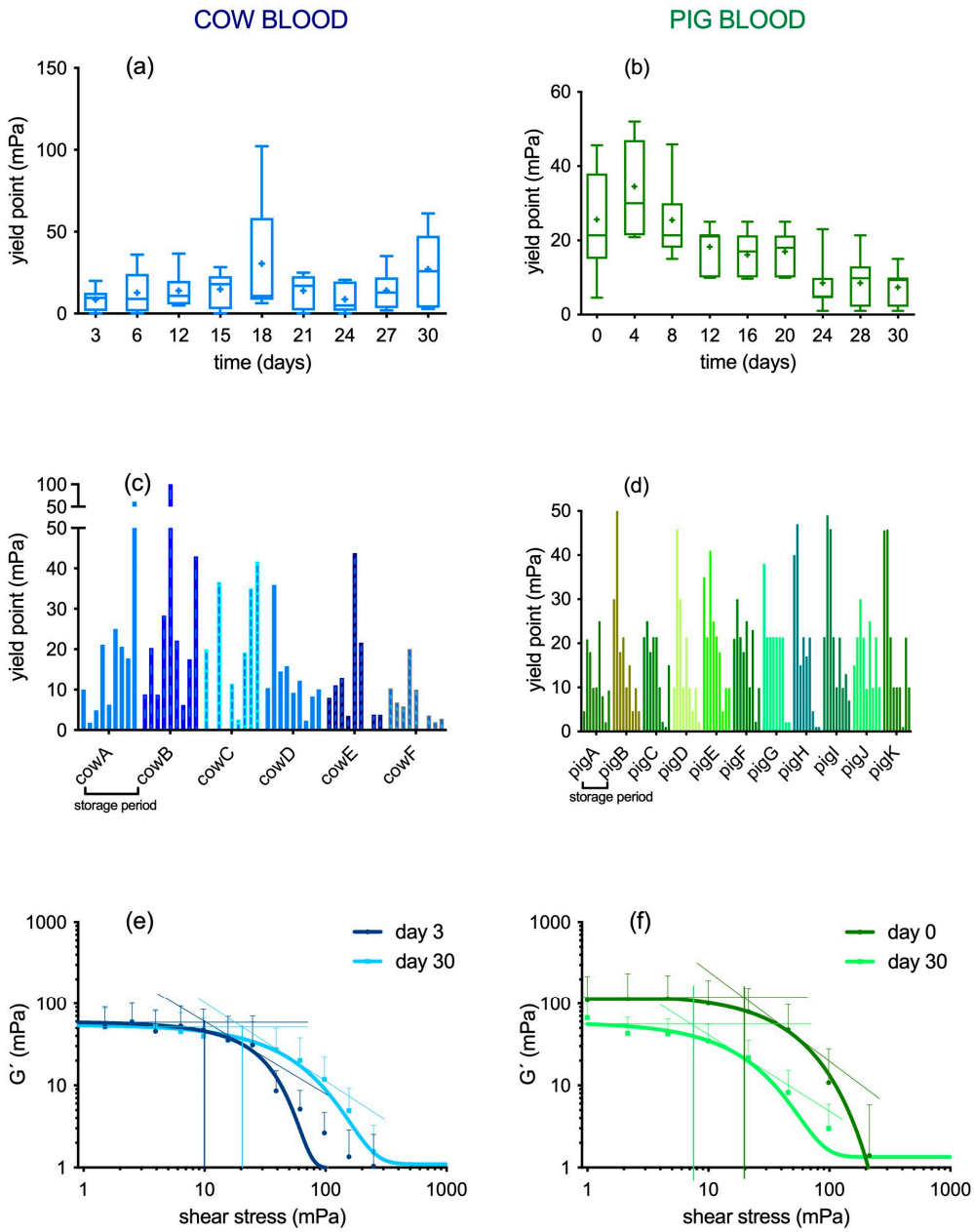

Figure 4. Yield points of bovine (n = 6, blue) and porcine (n = 11, green) whole blood obtained by amplitude sweep tests. (**a**,**b**): change in yield point with storage duration; the boxes represent median and interquartile range, asterisks show the mean value. (**c**,**d**): intraindividual variability of yield points during the time course; (**e**,**f**): yield point of fresh blood and aged blood on the 30th storage day: a quadratic regression curve interpolates the G′-values. The yield stress was obtained from the crossing point of the tangent that was drawn on the inflection point of this regression curve and crossed with a horizontal line through the first G′-values, which was extrapolated to the x-axis (method described in [12]).

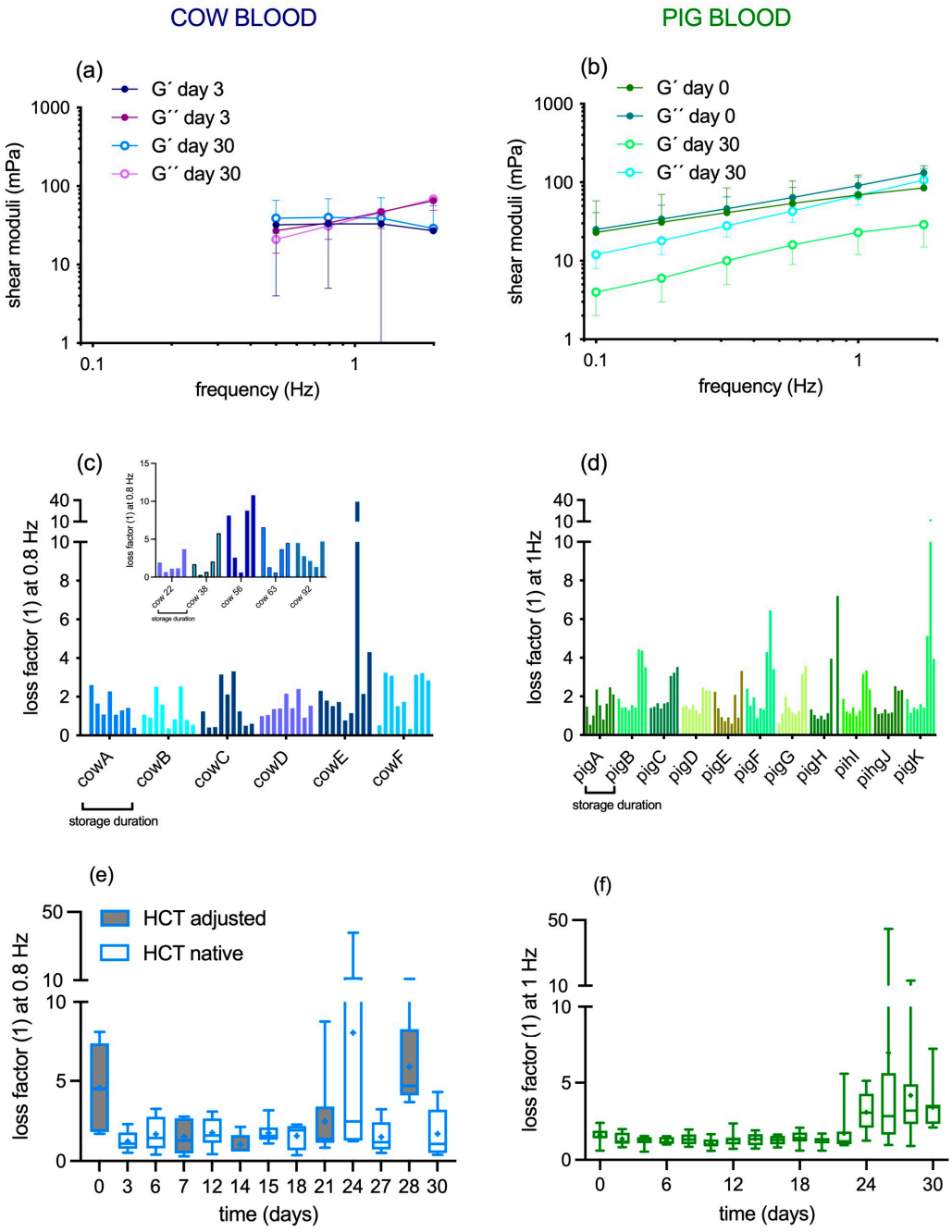

Figure 5. (**a**,**b**): Frequency spectrum of G′ and G″ of cow (n = 6, blue) and pig (n = 11, green) whole blood at start and end of storage. Pig blood fluidified, as indicated by the decrease in G′, whereas the shear moduli of HCT-native bovine blood hardly altered. (**c**,**d**): The intraindividual variability of loss factor (G″/G′) during the time course. The inset in Figure 2c shows the HCT-adjusted bovine sample. (**e**,**f**): The change in loss factor with storage duration. In porcine samples, loss factor values increased beyond day 22 due to hemolysis. In HCT-native bovine samples, loss factor values showed large errors but did not change with storage time (except one outlier at +24 days, cow E). In HCT-adjusted bovine samples, the loss factor decreased transiently around day 14 and returned afterwards. Boxes represent median and interquartile range; asterisks show the mean value. Values below the torque limit of the rheometer (1 µNm) are deleted from the spectrum in Figure 5a.

4. Discussion

We showed the preservation of the RBC count and the maintenance of the round shape of cow RBCs compared with pig RBCs together with the associated flow behavior of whole blood during 30 days of storage at equal conditions: the use of CPDA-1 as anticoagulant,

the refrigeration (4 °C) of native samples, mixing samples by gently turning the containers upside down for 10 min every 2 or 3 days. The finding that cow RBCs can better maintain their integrity is supported by their longer circulatory lifetime (130–160 days; compared with porcine RBCs (70–100 days [13])).

Storage lesions are well described [14,15]. Briefly, within the first week, the intracellular ATP levels fall, and the reactive oxygen species progressively oxidize the cellular structures. Lactate is generated, proteins are carbonylated, and the enzymes lose their function, which aggravates the metabolic emergency. Senescent signals appear on the cell surface; oxidized hemoglobin and membrane proteins aggregate or form heterologous clusters. Membrane clusters (e.g., band 3 protein, hemoglobin) can be expelled through blebbing but vesiculation also indicates the final stage of RBC damage and has several inflammatory, immunological, and hemostatic consequences [16–18]. The presence of spherocytes and echinocytes are thus the result of membrane loss and cytoskeleton re-arrangement. We show that cow RBCs shrink and become dense, which indicates vesiculation. However, the cells retain their round shape and there is no hemolysis present (except in one case). In contrast, all porcine samples become hemolytic. The reason for the preservation of bovine RBC's integrity has not been fully explored, although, the higher resistance of bovine RBCs against eryptotic shape changes at oxidative stress has been described earlier [4]. On a whole cell level, bovine RBCs are stiffer, which points to a better stabilization of the healthy membrane; when exposed to a Couette shear field, the cells show 7% reduced EI_{max} median value compared with porcine RBCs [3]. Details that may contribute to cell integrity during storage are the stability of cow band 3 oligomers [19] and the weak cohesion between the membrane phospholipids [20], which will stabilize the connection between cytoskeleton and membrane, and balance the membrane area between the transmembrane proteins. However, the metabolic situation is complex for the cells. A metabolomics study showed that stored cow RBCs accumulate pro-inflammatory prostaglandins faster than horse, dog, and donkey, and display the lowest glycolysis rate among these species, together with blockage at the level of fructose-1,6-bisphosphate, resulting in low ATP levels [21]. ATP is needed to drive the actin-protein 4.1R complex [22]. Upon phosphorylation, the complex loses its connection to the bilayer, allowing a higher strain at equal stress to the membrane. A loss of ATP thus reduces membrane deformation. Our study suggests that a stiff RBC membrane is protected from damage. The price to be paid is the higher flow resistance. As for the stability of the band 3 complex in stored porcine cells, the abundance of 2,3-BPG [23,24] can stabilize the membrane through quickly available deoxyhemoglobin binding to band 3, but this effect may have very little influence on long-term storage since intracellular 2,3-BPG is typically lost and samples remain O_2 deprived. Porcine RBCs lysed in our study, and as they often crenate ex vivo, we describe such finding as "pathologically normal deviations" of a healthy blood smear [25]. We found ghosts in the pig blood smears, which we did not find in the cow samples. This indicates that pig RBC membranes tend to be more vulnerable when stored.

Membrane shedding and crenation makes RBCs less deformable, which enhances their flow resistance. As a result, at fast shear flow, blood viscosity increases with storage time. This is described for several mammalian species [6,7,26,27]. Viscosity increased although HCT decreased in both species. However, the cause of the HCT decrease did not seem to play a role. The shrinkage of RBCs without lysis (observed in cow blood) or lysis with a parallel reduction in RBC count (observed in pig blood) have the same effect. In both circumstances, the flow resistance increases, by either the presence of dense and stiff cells, or the clustering of the remaining unlysed RBCs with themselves and the surrounding proteins (Figure 6).

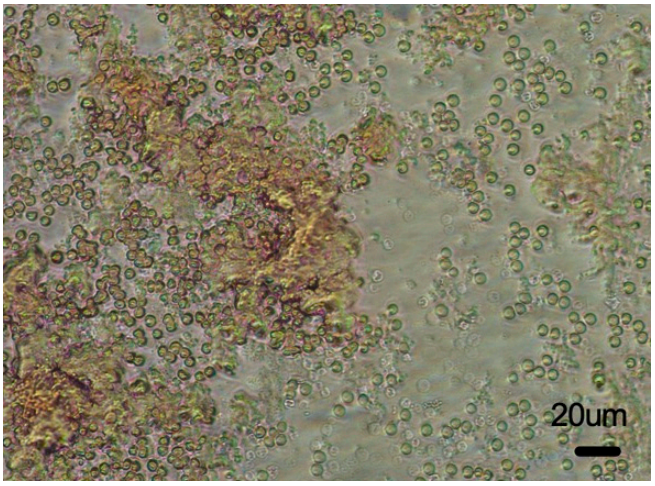

Figure 6. Blood smear from pig 1 (a different window of this smear is also shown in Figure 3) showing the clusters of cell debris and free hemoglobin.

Notably, a step in the timely increase in high shear viscosity (Figure 2b) can be seen in the pig samples after storage day 18 due to the onset of hemolysis around that date. Although high shear flow experiments provide information about the deformability of the components in which the RBCs make the largest contribution, the information about the extent to which a suspension is still homogeneous can be better obtained from low shear flow experiments. In simple shear, the degree of shear thinning gives insight into the correlation between the components of the suspension. A decrease in shear thinning towards the end of the storage period, as in pig and HCT-adjusted cow blood, is a sign of reduced interaction. In shear flow, such a suspension will structure and separate into phases [28,29]. At rest, the solid components sediment more quickly because their correlation is low, which facilitates the vertical fluid flow between them [5].

In small amplitude oscillating mode, the sample remains largely undisturbed by the flow in its quasi-static state. Fluidification is displayed by a reduction in G′ and yield stress. This occurred in porcine samples due to RBC destruction. In addition to the decrease in deformability as RBCs age, the reduction in the cohesiveness is the corresponding quality feature for the bulk sample. Stored porcine blood showed clear signs of ageing at both the cell and bulk levels. In contrast, the change in quality was less pronounced in HCT-native bovine blood. The yield stress varied intraindividually in the time course (compare with Figure 4c), so that the averaged shear elasticity (G′) and shear thinning values across all the samples remained unchanged during storage, but the high errors indicate the uncertainty of this assumption. The reason for this individual variability may be steric factors such as temporal changes in the size and random orientation of components—obviously clusters—in the gap. A further explanation is a more technical one that refers to the interface between sample and test substrate. If the adherence of blood to the metallic fixture changes with ageing (due to altered chemistry and conformation of plasma proteins and accumulating free hemoglobin), the recorded moments also vary because the shear stress cannot be always transmitted perfectly through the sample [30]. This simulates a change in bulk elasticity where there is none. When the HCT value in bovine samples is increased to compare it with the value of pigs, a pathological condition arises. The samples solidify after storage day 7, which means that structures must have formed in the blood while the cohesion of the sample remained intact. This could be due to an overlapping interaction of the components that form during aging, possibly as a result of protein degradation leading to condensation [31]. However, with increasing age, the cohesion of the samples decreases. In this respect, there is no difference between cow and pig samples. Both suspensions are

finally characterised by a shift towards Newtonian behavior and an increase in the loss factor, indicating suspension instability.

5. Conclusions

Cow blood is a good option when it must be stored long term. Pig blood is a good option when it can be consumed within 14 days (Table 2). A disadvantage of using bovine blood is the low physiological HCT, which will require the adjustment to human standards in many applications. However, elevating the HCT reduces shelf life significantly. A disadvantage of using pig blood is the risk of RBC lysis but hemolysis does not always occur. While hemolysis occurred in all porcine samples from the first cohort, in the second cohort, only one sample out of three became hemolytic. While the reason for this disparity is unclear, it could be related to factors not assessed in this study—e.g., bacterial sample contamination or the release of stress-related messenger molecules before or during phlebotomy. In any case, blood collection that is as stress-free as possible is to be welcomed for animal welfare reasons and will certainly improve the quality of the drawn sample. Further chances to maintain the integrity of porcine RBC is the addition of inosine, being the energy source for the RBCs [32]. For both blood types, storage containers other than 50 mL syringes may allow for a better resuspension of cell clusters in the regular intervals during storage, and using blood bags with low plasticizer release is preferred [33]. In this study, HCT-native samples were used, or the RBC concentrate gained after careful centrifugation was mixed with autologous plasma for HCT adjustment. Therefore, the blood cells remained in their physiological environment. In clinical practice, the RBCs are washed and stored in SAG-mannitol. However, the washing steps also change the interface between the cells and medium. RBCs that are suspended in autologous plasma are surrounded by an elastic surface layer [34], composed of plasma components. This halo protects the cells against damage. It is no longer present when the cells are suspended in a buffer medium; therefore, the exposure of the denuded membrane can contribute to the release of hemoglobin by the stored red cells [35]. All initial steps undertaken not only during but also before the blood is to be stored are crucial for the survival of the RBCs and the maintenance of a good suspension quality.

Table 2. Differences in blood properties and shelf life.

	Pig Blood	**Cow Blood**
Comparability of freshly drawn animal blood with human blood	good	poor
RBC integrity after 1 month storage	no	yes
Viscosity change at fast shear flow: HCT-native (HCT-adjusted)	+15%	+18% (+22%)
Viscosity change at slow shear flow: HCT-native (HCT-adjusted)	+15%	Unchanged (−26%)
Shear thinning change: HCT-native (HCT-adjusted)	−27%	−6% (−41%)
Sample cohesion change: HCT-native (HCT-adjusted)	−109%	Unchanged (+67% followed by—29% relative to start)
Shelf life: HCT-native (HCT-adjusted)	21 days	28 days (7 days)

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