The effect of preanalytical phase on the stability of osmotic fragility and morphological changes in bovine (Bos taurus) erythrocytes in cattle

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SUMMARY

Hereditary spherocytosis in cattle is a disorder resulting from a nonsense mutation in the SLC4A1 gene, which encodes band 3 membrane protein. This membranopathy leads to the production of spherically-shaped RBCs with reduced deformability and a shorter lifespan in circulation. Laboratory diagnosis of hereditary spherocytosis commonly relies on the erythrocyte osmotic fragility test (OFT), which is time- and labour-consuming and can be influenced by preanalytical factors. The aim of the study was to determine the effect of the preanalytical phase on the stability of osmotic fragility and morphological changes in bovine erythrocytes. Blood was collected from the tail vein of Holstein-Friesian cows (n = 20) into tubes with EDTA or lithium heparin. Erythrocyte resistance to haemolysis was measured by the osmotic fragility test immediately after blood collection and 24, 48 and 72 hours after storage at 4°C or at room temperature. Thin blood smears were also prepared, stained and examined for cell morphology.

The results showed that bovine erythrocyte osmotic fragility remains stable during 24-hour storage at 4°C irrespective of the anticoagulant used. This suggests that OFT can be performed the day after blood collection if blood is stored at 4°C. Bovine erythrocytes stored at room temperature were also found to have reduced osmotic resistance. However, heparin better preserves the osmotic resistance of bovine erythrocytes at room temperature. Erythrocytes undergo changes during storage. The microscopic examination of cell morphology revealed rapid transformation of bovine discocytes into various stages of echinocytes during the first three days of storage.

KEY WORDS: bovine RBCs, haemolysis, osmotic resistance, echinocytosis
INTRODUCTION

The mature erythrocyte in cattle is a biconcave cell with minimal central pallor. Bovine RBCs are generally uniform in shape and measure 5–6 µm in diameter; however, mild to moderate anisocytosis is quite common in cattle. The lifespan of the bovine RBC is approximately 130 days. The RBC membrane is unique with respect to phospholipid composition. The predominance of sphingomyelin over phosphatidylcholine is observed in the external hemilayer.

The assessment of RBC morphology provides valuable diagnostic information on the functioning of many organs and the causes of anaemia. For instance, the presence of poikilocytosis and anisocytosis may be a helpful diagnostic marker of advanced heart failure in dogs (Kumiega et al., 2020). Chmurska-Gąsowska et al. (2021) reported a higher percentage of lacrimocytes and schistocytes in female dogs with vaginitis. In cats, acanthocytes are often associated with liver disorders (Christopher and Lee, 1994). The observation of echinocytosis in dogs supports a clinical diagnosis of rattlesnake envenomation (Brown et al., 1996).

Hereditary spherocytosis (HS) is a heterogeneous disorder in which spherical RBCs (spherocytes) are produced. Spherocytes have reduced deformability and a shorter lifespan in circulation. This membranopathy results from disorders in RBC membrane structural proteins involved in the vertical interactions between the cytoskeleton and the lipid bilayer. In cattle, HS is caused by a nonsense mutation (CGA>TGA; Arg>Stop) in the SLC4A1 gene, which encodes band 3 protein (AE1, anion exchanger 1) and is inherited in an autosomal dominant manner (Inaba et al., 1996; Ito et al., 2007). Deficiency of this protein leads to erythrocyte instability, resulting in haemolytic anaemia and retarded growth. Spherocyte formation is also associated with bovine immune-mediated haemolytic anaemia.

Spherocytes appear on routine blood smears as microcytic, hyperchromic cells with no central pallor. Because cattle normally have RBCs with little central pallor, recognition of bovine spherocytes is more difficult than in species whose normal RBCs have distinct pale centres. Moreover, although spherocytes appear smaller than normal RBCs on a stained blood smear, in most cases they have normal volume, and their mean cell volume (MCV) remains unchanged. Therefore the determination of erythrocyte osmotic fragility is the most useful means of diagnosing hereditary spherocytosis (HS). The osmotic fragility of erythrocytes reflects their ability to withstand haemolysis due to the osmotic influx of water into the cell when placed in a hypotonic solution. The biconcave shape of normal RBCs promotes high osmotic resistance and allows them to increase their volume by about 70%. Spherocytes have an increased volume-to-surface area ratio and are particularly susceptible to osmotic lysis under exposure to hypotonic solution. Erythrocyte resistance to osmotic haemolysis is commonly measured by the osmotic fragility test (OFT), which is time- and labour-consuming and requires a relatively large volume of peripheral blood.

The preanalytical phase comprises sample collection, transport and storage. In haematological tests, to prevent blood from clotting, it is collected into test tubes with an appropriate anticoagulant, with which the tube is usually coated. After blood collection, it is important to store the samples at the appropriate temperature. The recommended storage temperature is 4°C, as this temperature ensures the stability of haematological parameters (Zini and ICHS, 2014). RBCs undergo a number of metabolic, structural and morphological changes during storage. Stored erythrocytes lose their normal biconcave shape, culminating in lysis of some of the cells. In in vitro-aged erythrocytes, depletion of ATP levels causes changes in the relative areas of the inner and outer leaflets of the
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Erythrocyte membrane. The expansion of the outer leaflet relative to the inner leaflet drives the formation of spicules on the membrane surface and the transformation of discocytes into echinocytes. Conversely, expansion of the outer leaflet leads to the formation of stomatocytes (Melzak et al., 2021). Given the above, the anticoagulated blood samples for hematological analysis must be processed within the recommended time. Moreover, poorly chosen anticoagulant concentrations can also cause disturbances in tonicity, cell swelling, haemolysis or crenation (Aryandi et al., 2019). The storage time of diluted blood samples with a dedicated diluent also affects hematological results (Bojarski et al., 2022). Hence, the preanalytical phase is considered to be the most vulnerable part of the entire testing process. The aim of this study was to determine the effects of preanalytical factors, i.e. the type of anticoagulant and storage conditions, on the osmotic fragility and morphology of bovine erythrocytes.

MATERIAL AND METHODS

Blood collection

The experiment was carried out on Holstein-Friesian cows (n = 20) aged from 25 to 46 months. Cows were kept in a free-stall barn and fed in the TMR (total mixed ration) system. Blood was collected from the tail vein (Vena coccygea mediana, s. Vena caudalis mediana) into blood collection tubes containing either EDTA (Kabe Labortechnik GmbH, Germany) or lithium heparin (Becton Dickinson, USA) as anticoagulant. The blood samples were collected during routine diagnostic procedures, so the study did not require ethics approval. After collection, the blood was gently mixed by inverting the tube several times and transported to the laboratory for analysis in a vaccine carrier with ice packs to maintain a temperature of 4°C. The blood samples were analysed within 4 hours after blood collection (day 0). The analysis included determination of erythrocyte osmotic fragility and preparation of thin blood films to evaluate morphological changes in erythrocytes. The blood samples were split into two portions; one was stored at 4°C, and the other at room temperature. The analysis was repeated at 24-hours intervals until the third day (day 1 – day 3).

Determination of erythrocyte osmotic fragility

Erythrocyte resistance to haemolysis was measured by the osmotic fragility test (OFT). The test was performed by adding 10 µl of well-mixed blood to tubes containing 1.5 ml phosphate buffered saline (pH 7.4) at serial concentrations ranging from 10 to 150 mM. The mixtures were allowed to stand at room temperature for 30 minutes, followed by centrifugation (MPW-55, MPW Med. Instruments) for 5 minutes at 3500 rpm. The supernatant was transferred from tubes to a 96-well plate, and the haemoglobin concentration was measured spectrophotometrically at a wavelength of 540 nm using the EPOCH2 microplate reader (BioTek Instruments Inc., USA).

The supernatant from the 150 mM concentration was used as a blank control to eliminate undesired absorbance from blood plasma components. The percentage of haemolysis in each concentration of saline was calculated assuming 100% haemolysis in the concentration with the highest absorbance. Fragility curves were prepared. Osmotic fragility was expressed as the highest concentration of saline at which lysis is just detectable (minimum resistance), the highest concentration of saline at which lysis appears to be complete (maximum resistance), and the concentration of saline causing 50% lysis (MCF, mean corpuscular fragility).
Microscopic examination and cell counting

Blood smears were prepared from each blood sample and stained by the May-Grünwald-Giemsa staining method. The stained blood smears were examined for erythrocyte morphology using a computerized image-analysis system (MultiScanBase v. 14.02, Computer Scanning System, Warsaw, Poland). Cells were counted on representative single-layer visual fields in which blood cells just touched one another at the edges and did not overlap. At least 1000 RBCs were analysed in each blood smear to determine the presence of poikilocytic erythrocytes. The number and type of poikilocytes were recorded as percentages of red blood cells.

Statistical analysis

Statistical analysis of the data from the experiments was performed using Statistica 13.3 (Tibco Software Inc., USA). The results for minimum and maximum resistance were analysed by Friedman repeated measures analysis of variance by ranks. The results for mean corpuscular fragility (MCF) and cell counting were analysed by repeated measures ANOVA followed by Tukey’s post-hoc test. The assumptions of normality and sphericity were previously tested using the Shapiro–Wilk test and Mauchly’s test, respectively. Values are expressed as the mean ± SEM and considered significantly different at p < 0.05. Figures were prepared using Grapher 10.3 (Golden Software Inc., USA).

RESULTS AND DISCUSSION

Erythrocytes are the blood cells that are most susceptible to damage. Biomechanical, biochemical, and immune reactions occur during blood storage, leading to morphological changes in the cell. To reduce these processes, it is important to select an appropriate anticoagulant. The accuracy of haematological test results can be influenced by the storage conditions of blood samples (Makroo et al., 2011; Faggio et al., 2013; Aryandi et al., 2019).

The main conclusion that we can draw from our results is that there is no need to perform the osmotic fragility test on the day of blood collection. Keeping blood samples at 4°C preserved their resistance to osmotic haemolysis during 24-hour storage, irrespective of the anticoagulant used. The three parameters examined, i.e. minimum resistance (Fig. 1), maximum resistance (Fig. 2) and mean corpuscular fragility (MCF; Fig. 3), remained stable during this time (day 1). After 48 hours, the minimum and maximum resistance did not change relative to the day of blood collection, but there was a significant (p < 0.05) increase in MCF (Fig. 3), which means that the osmotic fragility of bovine erythrocytes should not be assessed on the second day after blood handling.
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Figure 1. Minimum osmotic resistance of erythrocytes in blood samples collected into tubes with EDTA (A, C) or heparin (B, D) and stored at 4°C (A, B) or room temperature (C, D). Each value represents the mean ± SEM. Values marked with different letters differ significantly (p < 0.05).

Figure 2. Maximum osmotic resistance of erythrocytes in blood samples collected into tubes with EDTA (A, C) or heparin (B, D) and stored at 4°C (A, B) or room temperature (C, D). Each value represents the mean ± SEM. Values marked with different letters differ significantly (p < 0.05).
The MCF of erythrocytes in heparinized blood and EDTA-anticoagulated blood showed a similar pattern of changes during three days of storage at 4°C (Fig. 3). MCF values were lower for heparin than for EDTA, but no statistically significant differences were found between these anticoagulants. However, heparin better preserves the osmotic resistance of bovine erythrocytes at room temperature, as MCF was statistically higher (p < 0.01) in EDTA-anticoagulated blood at day 3 (Fig. 3). Moreover, refrigerated storage kept the minimum resistance unchanged for three days in heparinized blood samples (Fig. 1B), whereas in EDTA blood samples this parameter was stable for only two days (Fig. 1A). These results suggest that osmotic fragility can be assessed within 24 hours after blood collection if samples are stored at 4°C. In addition, heparin should be the anticoagulant of choice for OFT of bovine erythrocytes. Bovine erythrocytes seem to be more osmotically fragile than human RBCs, as Salvagno et al. (2020) showed that blood samples should not be stored at 4°C for more than two days before osmotic fragility is tested.

The results confirm that erythrocytes in blood collected in EDTA tubes may be more susceptible to haemolysis in a hypotonic environment compared to heparin, which is consistent with previous studies (De Caro et al., 1991; Lektib et al., 2016). When erythrocytes are placed in a hypotonic solution, their volume suddenly increases. This is followed by a gradual return to normal volume. This mechanism is associated with an increase in cell membrane permeability. The cell membrane permits the outflow of intracellular osmolytes, reducing the force with which water flows into the erythrocyte. Calcium ions play a crucial role in this mechanism (Pierce and Politis, 1990). The use of EDTA as a chelating agent can result in the loss of calcium ions and may impede the return of erythrocytes to their normal volume, which in turn can cause hypotonic stress and increased haemolysis. However, this mechanism is not observed in heparinized blood samples (Kafka and Yermiahu, 1998).
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The MCF of erythrocytes in blood stored at room temperature was significantly higher than that of blood samples stored at 4°C (Fig. 3). This means that the higher temperature increases the susceptibility of erythrocytes to haemolysis in hypotonic solutions. In this experiment, a temperature of 4°C maintained osmotic resistance for 24 hours, whereas storage of blood at room temperature leads to a reduction in erythrocyte osmotic resistance after 24 hours.

Stored erythrocytes undergo changes during the storage period. In the blood smear examination, negligible amounts of stomatocytes (< 0.5%), dacrocyes (< 0.1%) and keratocytes (< 0.1%) were detected (data not shown). The microscopic examination of cell morphology revealed the transformation of bovine discocytes into echinocytes, which rapidly occurs during the first three days of storage. Cell counting showed marked echinocytosis with statistically significant differences between various stages of echinocytes (burr cells) observed in the blood on different days. Type I echinocytes were seen as RBCs with ripples around the edges but no bumps on the top surface. RBCs with equally spaced blunt spicules were classified as type II echinocytes. Type III echinocytes appear on a blood smear as spherical RBCs with equally spaced pointed projections (Fig. 4).

Figure 4. Poikilocytic forms of bovine erythrocytes: A) normal red blood cell (red arrow), type I echinocytes (yellow arrows); B) type II echinocytes (blue arrows); C) numerous type III echinocytes (green arrows). Peripheral blood smear, May-Grünwald-Giemsa staining method, magnification 400×.
The results of echinocyte counting are shown in Figure 5. The first day revealed marked echinocytosis with the presence of type I echinocytes in the blood samples stored at 4°C. The number of type I echinocytes increased continuously during storage, reaching almost 30% of total RBCs in EDTA-anticoagulated blood and over 70% of total RBCs in heparinized blood on day 3. Surprisingly, the number of type I echinocytes increased more rapidly in blood with heparin, which is inconsistent with the higher osmotic resistance in heparinized blood.

At room temperature, the transformation of discocytes through successive stages into type III echinocytes was clearly visible (Fig. 5C, 5D). In EDTA blood samples, type I echinocytes dominated on day 1, but from day 2 type II echinocytes constituted a greater proportion of RBCs. In heparinized blood samples, continuous changes were observed as well. Type II echinocytes dominated on days 1 and 2, but on day 3 the number of type III echinocytes surpassed the other types of burr cells. To sum up, the formation of echinocytes was significantly higher in blood stored at room temperature, and changes in erythrocyte shape occurred more rapidly in heparinized blood. The results suggest that the change in the shape of erythrocytes stored for a long time significantly disrupts their resistance to hypotonic solutions, causing them to undergo haemolysis earlier than normal erythrocytes. The study confirms that erythrocytes lose their osmotic resistance over time, which is associated with the formation of echinocytes. In the study of human erythrocytes, morphological changes such as spherocytosis, echinocytosis and spheroechinocytosis have also been observed 24 hours after blood collection (Antwi-Baffour et al., 2013).

Figure 5. The percentage of poikilocytic erythrocytes in EDTA blood samples stored at 4°C (A) or room temperature (C) and heparinized blood samples stored at 4°C (B) or room temperature (D). Each value represents the mean ± SEM. Values marked with different letters differ significantly (p < 0.05).
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Erythrocytes are considered to be the most fragile blood cells and the most prone to intra- or extravascular destruction. Proper handling of blood samples is particularly important, as the anticoagulant, temperature, and storage time can all have negative effects on erythrocytes and lead to changes in the erythrocyte membrane and its breakdown. This can cause unreliable results of the osmotic fragility test of erythrocytes, potentially leading to a misdiagnosis of haemolytic anaemias, especially spherocytosis.

CONCLUSION

As mentioned above, the preanalytical phase plays a crucial role in laboratory diagnostics. Our results showed that bovine erythrocyte osmotic fragility remains stable during 24 hours of storage at 4°C, irrespective of the anticoagulant used. This suggests that OFT can be performed on the day after blood collection if blood is stored at 4°C. The study confirms that erythrocytes lose osmotic resistance over time, which is associated with the formation of echinocytes.

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