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Inverse relationship between the number of blood draws and the osmotic fragility of sheep erythrocytes

Relación inversa entre el número de extracciones sanguíneas y la fragilidad osmótica eritrocitaria en carneros

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ABSTRACT

Sheep blood is an important biological reagent for clinical and microbiological laboratories. It is essential to maintain the cellular integrity of its erythrocytes when used in hemolytic reactions of diagnostic protocols. The objective of this study was to determine the relationship between the number of blood draws in rams and erythrocyte osmotic fragility, and its correlation with blood storage time at (2-8) °C, with its thermal robustness for 16 h at laboratory temperature (22-25) °C, and with the welfare and behavior of the rams. The experiments revealed that erythrocyte osmotic fragility is inversely related to the number of blood draws. Hemoglobin concentration remained within the normal range for up to six blood draws, and indicators of animal welfare and behavior did not change. Erythrocytes maintained their osmotic integrity for 42 days of storage at (2-8) °C and for up to 16 h at (22-25) °C. This work has demonstrated, for the first time, the resistance of sheep blood to hemolysis after repeated blood draws from the same animal or exposure to suboptimal temperatures, confirming its use as a reliable reagent in routine diagnostic procedures.

Palabras clave: animal welfare; blood storage; sheep blood

RESUMEN

La sangre de carnero es un reactivo biológico importante para los laboratorios clínicos y microbiológicos. Es esencial mantener la integridad celular de sus eritrocitos cuando se utiliza en reacciones hemolíticas en protocolos de diagnóstico. El objetivo del presente trabajo fue determinar la relación entre el número de extracciones de sangre en carneros y la fragilidad osmótica eritrocitaria, y su correlación con el tiempo de almacenamiento de la sangre a (2-8)°C, con su robustez térmica 16 h a temperatura de laboratorio (22-25) °C, y con el bienestar y el comportamiento de los carneros. Los experimentos revelaron que la fragilidad osmótica eritrocitaria está inversamente relacionada con el número de extracciones de sangre. La concentración de hemoglobina se mantuvo en el rango normal hasta seis extracciones de sangre, y no cambiaron los indicadores de bienestar ni del comportamiento animal. Los eritrocitos mantuvieron su integridad osmótica durante 42 días de almacenamiento a (2-8) °C y hasta 16h a (22-25) °C. Este trabajo ha demostrado, por primera vez, la resistencia de la sangre de carnero a la hemólisis, tras repetidas extracciones de sangre al mismo animal o exposición a temperaturas subóptimas, lo que la confirma como un reactivo fiable en los procedimientos diagnósticos rutinarios.

Keywords: bienestar animal; almacenamiento de sangre; sangre de carnero

1. INTRODUCTION

Sheep blood is a biological reagent of utmost importance as a nutrient supplement for the preparation of agar-based culture media, preclinical testing and biomarker development for diagnostics (1, 2). It has been used in procedures such as exploring complement functional activities and for hemolytic tests, and is considered the gold standard reagent for defining hemolytic reactions and allowing better visualization of hemolysis (3). In the hemolytic test, some bacteria, such as *Streptococcus pyogenes*, produce hemolysins that destroy erythrocytes (4). If the sheep blood used as reagent is already hemolyzed, it would not be possible to distinguish between hemolysis caused by the bacteria and that which was already present. Furthermore, it is essential to maintain the cellular integrity of the erythrocytes to correctly assess the types of hemolysis and to preserve their surface antigens, which are essential for agglutination tests and other immunoassays (5). Therefore, using non-hemolyzed sheep blood guarantees reliable, reproducible, and clinically useful results.

Basic science studies have suggested that, at least from a molecular perspective, long-term stored blood products are qualitatively different from short-term stored blood products, as stored erythrocytes undergo biochemical, morphological, and functional changes (6). These modifications have been termed "storage lesions," and some of their causes include accumulation or depletion of metabolites, for which additive solutions have been incorporated, and oxidative damages, which have not been studied in depth (7). One such blood storage lesions is the erythrocyte hemolysis (8).

Numerous intrinsic and extrinsic factors influence the osmotic fragility of red blood cells. Extrinsic factors involve the type, ionic strength, and pH of the incubation medium, the anticoagulant and the length of blood storage, while intrinsic factors include sex, age, breed, and genetic factors, among others (9).

The osmotic fragility test is conventionally used to assess the resistance of erythrocytes to hemolysis, using a series of hypotonic sodium chloride (NaCl) solutions (10). This analytical technique is currently widely applied in clinical laboratories to evaluate the erythrocyte deformability and viability under different storage conditions (11, 12).

The stability of ovine erythrocytes for laboratory use has been known since the beginning of the twentieth century (13). However, to date, no studies have been conducted to define the influence of the repeated blood draws, from the same animal, on sheep blood stability, as measured by erythrocyte osmotic fragility. It is also unknown how the repeated blood draws affects the osmotic fragility of the stored blood and the welfare of the blood donor sheeps.

The objective of this work was to determine erythrocyte osmotic fragility in blood donor sheeps with different numbers of blood draws. The effect of the repeated blood draws on the erythrocyte osmotic fragility and on animal welfare was studied, taking into account the biological variability among sheeps. Blood stability was also monitored based on storage time (2-8) °C and the number of blood draws. Thermal robustness of blood was evaluated according to its permanence during 16 h at laboratory temperature (22-25) °C and to the number of blood draws.

2. MATERIALS AND METHODS

2.1. Animals

Healthy sheeps were selected from the dark red Pelibuey breed, two-year-and-six-month-old, weighing between 50 and 70 kg. They were kept in semi-stable conditions with access to pasture and supplementary food, consisting of a concentrate-based diet that included corn, soybean meal, alfalfa, methionine, lysine, monocalcium phosphate, calcium carbonate, common salt, vitamin-mineral premix and glucose.

2.2. Sheep blood collection

Blood draws were performed according to the WVU IACUC Guidelines (14). Blood collection to bags was performed on a surgical table. During the procedure, the animals were properly restrained. Bags used for blood collection and storage were made of polyvinyl chloride (PVC) and the plasticizer DEHP (di-2-ethylhexyl phthalate). Each CPD (citrate-phosphate-dextrose) bag contains, per 100 mL of water for injection: 0.299 g of anhydrous citric acid; 2.630 g of sodium citrate dihydrate; 0.222 g of sodium phosphate monohydrate; and 2.550 g of dextrose monohydrate. Each bag used contained 63 mL of anticoagulant for the collection of 450 mL of whole blood.

To evaluate the effect of sheep biological variability on erythrocyte osmotic fragility, measured within 3 h of blood collection, the animals remained stationary. Blood was not collected in bags, but in Vacuette® tubes. The blood collected form each sheep was carefully transferred to 2 mL Vacuette® tubes, type K3E (K3EDTA), ensuring that it flowed slowly along the tube wall to avoid hemolysis. Approximately 1.96-1.98 mL of blood was withdrawn per 0.02-0.04 mL of anticoagulant.

2.3. Erythrocyte osmotic fragility test

To quantify erythrocyte osmotic fragility, a modification of the Aldrich et al. (15) method was performed, using a 10% (w/v) NaCl solution dissolved in phosphate buffer (10 mM; pH 7.4). This NaCl solution was diluted with phosphate buffer to prepare nine solutions with different NaCl concentrations: 0.9%; 0.8%; 0.7%; 0.6%; 0.4%; 0.35%; 0.3%; 0.2% and 0.1%. The solution 0.00% consisted of 1 mL of distilled water. All solutions were prepared in duplicate in Eppendorf® tubes. Each tube, with 1 mL of each solution separately, was vortexed for 10 seconds, and 26 µL of previously homogenized sheep blood was added by careful repeated inversions. This final mixture was homogenized again following the previous procedure and left to stand for 30 min at room temperature (22-25 °C).

After this time, each tube was homogenized again and centrifuged at 3000 rpm (604 x g) for 5 min at 20°C. Then, 100 µL of each sample was applied in triplicate to a reading plate. The absorbance at 530 nm (A530nm) was measured on a SUMA plate reader (TecnoSUMA International, Havana, Cuba).

The percentage of hemolysis was calculated using the following formula:

$$Hemolysis\,(\%) = \frac{A(sample) - A(0.9\%)}{A(H_2O) - A(0.9\%)}\ x\ 100$$

Where:

A (sample): the average A530nm of the blood sample at each NaCl concentration

A (0.9%): the A530nm of the sheep blood sample plus 0.9% NaCl (maximum NaCl concentration used in the test)

A (H_2O): the A530nm of the total hemolysis of the sheep blood sample in distilled water (solution 0.00%)

To graph the percentage of hemolysis as a function of NaCl concentration, a sigmoid curve was fitted to a five-parameter logistic function using Graph Pad Prism, version 8.0.1. The NaCl concentration at which 50% of the erythrocytes are hemolyzed (C-50), a parameter obtained directly from the program output, was considered the indicator of osmotic fragility.

2.4. Evaluation of the influence of the number of blood draws on the erythrocyte osmotic fragility and on animal welfare

In this study, two groups of sheeps with repeated blood draws were used. Group 1 consisted of four sheeps with the fewest blood draws: three with three draws and one with two. Group 2 consisted of six sheeps with the highest number of blood draws: four sheeps with five draws and two sheeps with six. In all cases, blood was collected in Vacuette® tubes for the osmotic fragility test.

General indicators of welfare (body condition, feed intake and physical activity), behavior (signs of stress, aggression or apathy), as well as hemoglobin, quantified according to Drabkin and Austin (16), were used to assess the impact of the repeated blood draws on donor sheeps.

2.5. Analysis of the effect of sheep blood storage time at (2-8) °C and number of blood draws on erythrocyte osmotic fragility

Three bags of blood corresponding, respectively, to one sheep subjected to two blood draws, another to four, and the last to six blood draws, were used. The erythrocyte osmotic fragility of blood from each bag was measured at 0, 7, 14, 21, 30, and 42 days of storage at (2-8) °C.

2.6. Assessment of the thermal robustness of sheep blood after 16 h at 22–25 °C

The thermal robustness of blood from two sheeps with two and six blood draws, respectively, was analyzed. The blood had been stored for 15 days in bags at (2-8) °C. Afterward, 4 mL of blood from each bag was poured into Eppendorf® tubes. These were left on the laboratory bench at (22-25) °C from 17:00 until 9:00 the following morning (total: 16 h). The blood from each sheep that remained in the bags at (2-8) °C was used as a control for this experiment.

The osmotic fragility test was applied to blood maintained at (22-25) °C for 16 h, and its results were compared with those of the control stored at (2-8) °C.

Statistical Analysis

Experiments evaluating the effect of sheep blood storage time (2-8) °C on erythrocyte osmotic fragility, and the thermal robustness of sheep blood were analyzed by two-way ANOVAs. Multiple comparisons of means were performed using the least significant difference test, using the Bonferroni correction. Effect size was measured using the partial Eta-squared test.

The influence of the number of blood draws on erythrocyte osmotic fragility and hemoglobin concentration, taking into account biological variability, was analyzed by an independent samples t-test. Effect size was assessed using Cohen's d statistic.

Statistical analyses were conducted with the SPSS 25 software (Statistical Package for the Social Sciences; SPSS Inc., Chicago, IL). All statistical inferences were performed with a significance level of $\alpha = 0.05$.

3. RESULTS AND DISCUSSION

Influence of the number of blood draws on erythrocyte osmotic fragility and on animal welfare

In this experiment, two groups of sheeps were evaluated with differences in the number of extractions performed on the animals included in both of them. It was possible to evaluate the influence of the number of draws on osmotic fragility, taking into account the biological variability between sheeps. This allows for the inclusion of the biological variability in the experimental error and yielding results that are representative of the study population.

In the evaluation of the impact of repeated blood draws on the erythrocyte osmotic fragility (Fig. 1A), significant differences were observed between both groups ($p = 0.028$). The effect size of the number of blood draws on the NaCl concentration that produced 50% hemolysis, measured by Cohen's d ($d = 1.73$), was very high, indicating that the number of blood draws is a factor that has a significant impact on the erythrocyte osmotic fragility. For the group with the fewest blood draws, the average C-50 was significantly higher (0.58%) than for the group with the highest number of blood draws (0.47%).

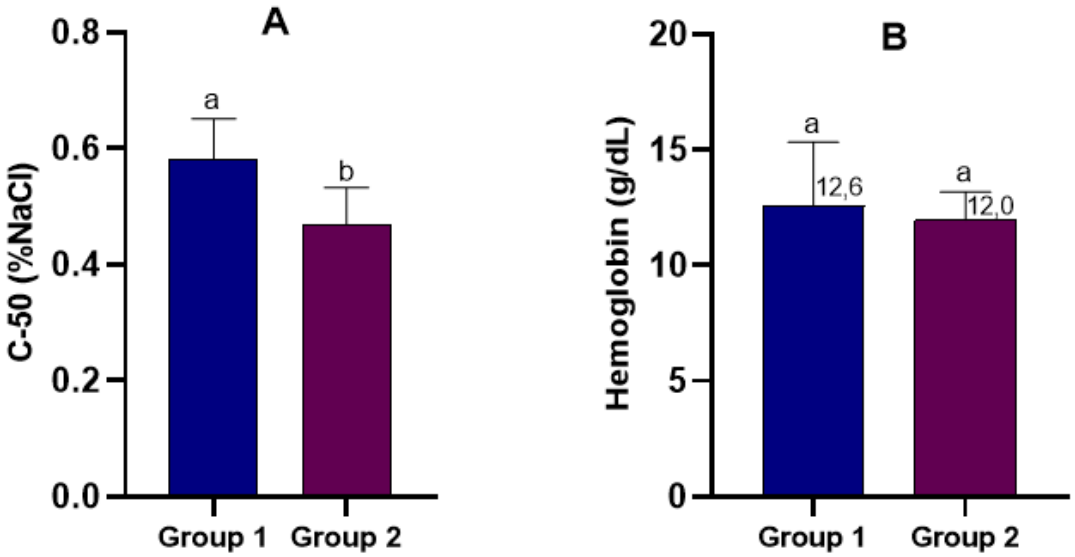

Figure 1. Effect of the number of blood draws on erythrocyte osmotic fragility **(A)** and hemoglobin concentration **(B)**. C-50 is the NaCl concentration at which 50% of the erythrocytes are hemolyzed. Each column represents the arithmetic mean plus standard deviation. Group 1: four sheeps with 2 to 3 blood draws. Group 2: six sheeps with 5 to 6 blood draws. Different letters in the columns of the graphs represent significant differences between groups, according to the independent samples t-test ($p < 0.05$)

To our knowledge, this is the first report in sheep of the effect of the number of blood draws on the erythrocyte osmotic fragility, particularly which they can become more resistant to osmotic hemolysis with successive blood draws.

This could be due to physiological adaptation mechanisms to the repeated blood draws, involving changes in cell membrane composition or modifications in the response of the hematopoietic system. It has been found that the high concentration of total lipids, cholesterol, proteins, sphingomyelin and phosphatidylcholine in camel erythrocyte membranes confers the lowest osmotic fragility, compared to a long list of animal species that have lower concentrations of these components (17). It could be thought that the increased number of blood draws in the sheep is related to the increased concentrations of these components of the erythrocyte membrane, and that this gives it greater resistance to osmotic changes.

No alterations were observed in the general welfare indicators of the animals. Assessing the body condition of sheeps is a key tool for monitoring their nutritional status, health and welfare (18). All animals included in the study maintained good body cover and were free of excess fat. No signs of stress, aggression or apathy were observed in either group. Marin et al (2023) (19) stated that the handling required for animal blood collection can have a significant impact on the condition and responses of donor animals. Sheep erythrocytes are sensitive to hemolysis under stress conditions (20), for example under situations of metabolic stress with high energy demands (21). According to the results of the present work, the increase in resistance to hemolysis, with increasing number of blood draws up to six, indicates that these successive blood draws did not represent a stress that triggered hemolysis in the blood from the sheeps participating in the study.

The hemoglobin concentration remained within normal ranges (Fig. 1B) and there were no significant differences between the two groups of sheeps ($p= 0.636$ and a low Cohen's d of 0.32). The number of blood draws, up to six, did not affect the normal values for this blood parameter.

All these results indicated that the repeated blood draws, which were performed according to the WVU IACUC Guidelines (14), did not affect the welfare of the sheeps. However, other physiological indicators, such as erythrocyte count and morphological evaluation, could be analyzed in future studies, to detect

possible changes in red blood cell ontogeny and elimination. It is also advisable to monitor the nutritional status of the sheeps by measuring serum iron and total plasma protein concentrations, considering that blood regeneration depends on adequate nutrient intake and assimilation in animals.

Effect of sheep blood storage time at (2-8) °C and the number of blood draws on erythrocyte osmotic fragility

To our knowledge, this work also reports for the first time, the influence of the interaction between the number of blood draws and the storage time of blood at (2-8) °C on the osmotic fragility of sheep erythrocytes. Although the variable with the greatest effect size on C-50 was the storage time (partial Eta-squared= 0.978), it is important to note that the interaction between the two factors was significant (p= 0.002), meaning that they do not act independently on erythrocyte osmotic fragility and that the interactive effect size is high (partial Eta-squared= 0.723).

The blood of the sheeps participating in the study showed a significant decrease (p=0.000) in C-50 between day 0 and day 7 of storage at (2-8) °C. This means that erythrocytes, after one week at this temperature, become more resistant to hemolysis. Reed [13] observed this same phenomenon, but after ten days.

In general, from 7 to 42 days of storage, the average of C-50 values remained constant (p≥0.098). This same behavior up to day 82 was described by Reed (13), but without taking into account the number of blood draws from the sheeps.

At day 0, the average of C-50 values among the three sheeps were different (p≤0.045). The highest average C-50 was found in the sheep with two blood draws, and the lowest in the sheep with six blood draws (Fig. 2). This result is consistent with those of the previous experiment, supporting the fact that the greater the number of blood draws, the lower the osmotic fragility of erythrocytes.

The outcomes of this experiment indicates that, regardless of the differential behavior of erythrocyte osmotic fragility in relation to the number of blood draws, sheep blood maintains its osmotic integrity for at least 42 days. This finding provides important information on the stability of sheep blood stored for use as a biological reagent in laboratories.

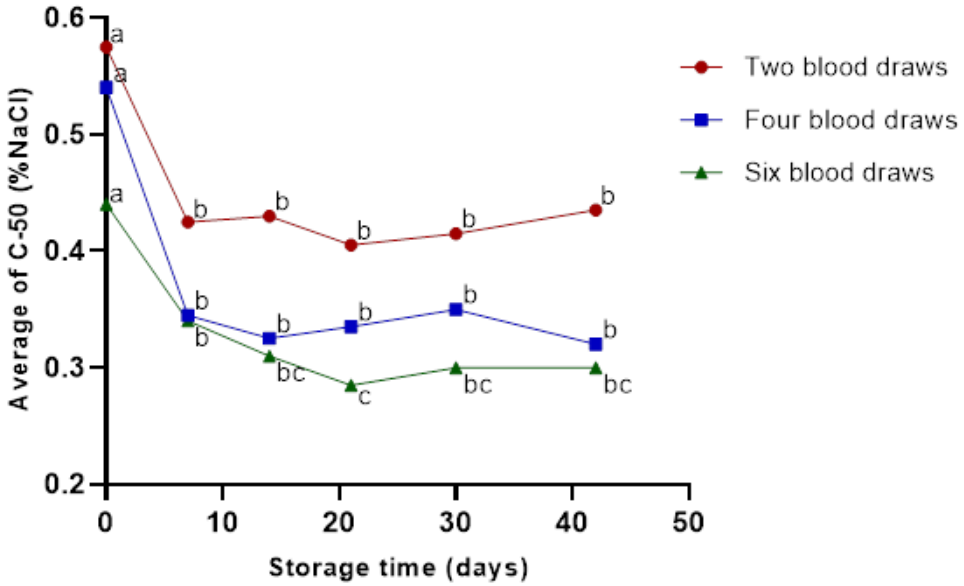

Figure 2. Influence of storage time (2-8) °C and the number of blood draws on erythrocyte osmotic fragility. C-50 is the NaCl concentration at which 50% of the erythrocytes are hemolyzed. Different letters denote significant differences between storage times for each curve, according to a two-way ANOVA with multiple comparisons of means, using the least significant difference test, and Bonferroni correction ($p < 0.05$)

Thermal robustness of sheep blood after 16 h at 22–25 °C

Robustness is the ability of an analytical procedure to maintain its measurements unchanged when small, deliberate changes are made to the experimental conditions (22). Biological reagents, such as blood, may also experience alterations in their physical, chemical and biological integrity when subjected to unexpected practical changes. For example, in clinical and microbiological laboratories that process many blood samples, analytical processing may experience delays, resulting in longer storage times for samples at laboratory temperature, which is not the optimal storage temperature. No specific studies have been published analyzing the erythrocyte osmotic fragility of sheep blood stored at room temperature as a reagent.

This experiment was conducted to evaluate the robustness of sheep blood based on the time spent at laboratory temperature (22–25 °C). The 16-h blood retention time was chosen because it represents a realistic situation that can occur in the laboratory, where samples may remain on the laboratory bench from the end of the workday until the following morning.

No significant differences (p= 0.230) were observed in the C-50 of the control blood (2-8 °C) and the samples that remained at 22-25 °C for 16 h (Fig. 3A).

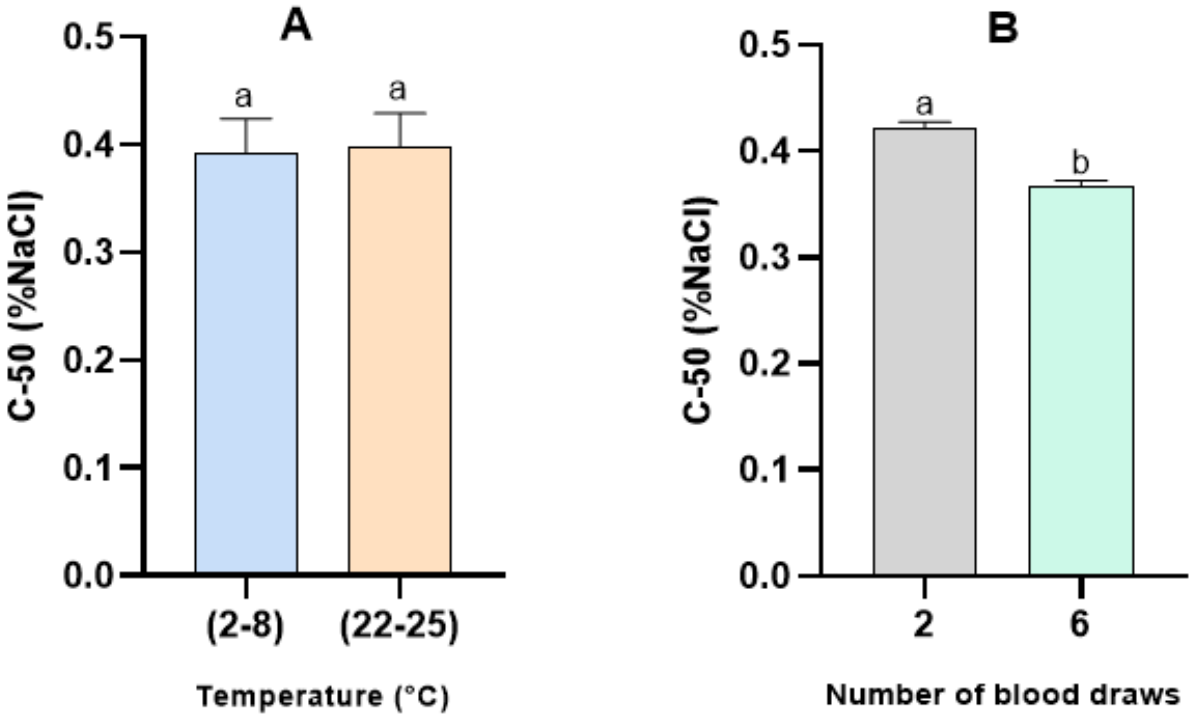

Figure 3. Thermal robustness of sheep blood **(A)** with different numbers of blood draws **(B)**. C-50 is the NaCl concentration at which 50% of the erythrocytes are hemolyzed. Different letters denote significant differences between the factors evaluated, according to a two-way ANOVA with multiple comparisons of means, using the least significant difference test with the Bonferroni correction ($p<0.05$)

As in the previous experiments, significant differences (p= 0.0001) in C-50 were maintained between sheeps with different numbers of blood draws (Fig. 3B).

However, in this case, the interaction between this factor and the time the blood remained at laboratory temperature did not have a significant impact (p= 1.000) on osmotic fragility. This indicates that the blood maintains its robustness for up to 16 h after remaining at (22-25) °C, regardless of the number of draws applied to the donor sheep.

These results have demonstrated that the resistance of sheep blood to hemolysis, even after successive blood draws from the same animal or prolonged exposure to suboptimal environmental conditions, makes it a reliable reagent in clinical and microbiological laboratories.

CONCLUSIONS

All experiments performed in this work suggest that the osmotic fragility of sheep erythrocytes is inversely related to the number of blood draws. Sheep erythrocytes increase their resistance to hemolysis during the first week of storage at (2-8) °C. Stored blood is less hydrolyzed if it comes from a sheep with more blood draws. General welfare indicators and animal behavior are not affected by increasing of blood draws. Sheep blood is a robust biological reagent that can remain at (22-25) °C for up to 16 h, without changes in its erythrocyte osmotic fragility.

This work has demonstrated, for the first time, the resistance of sheep blood to hemolysis, even after repeated blood draws from the same animal or exposure to suboptimal temperatures, making it a reliable biological reagent in routine diagnostic procedures.

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CONFLICT OF INTERESTS

The authors have no conflict of interests to declare concerning the authorship or publication of this article.

AUTHORS CONTRIBUTIONS

Luis Miguel Águila-Pérez: Data curation, Investigation, Methodology, Resources, Software, Visualization, Writing – original draft. Angel Aquino-Perna: Conceptualization, Investigation, Methodology, Project administration, Supervision. Maylin Pérez-Bernal: Visualization, Software, Writing – original draft, Writing – review & editing. Adria Fernández-Pérez: Investigation. Carlos Hernández-Díaz: Formal analysis, Investigation, Methodology, Supervision, Validation, Writing – original draft.

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