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Use of capillary electrophoresis–isoelectric focusing for the determination of bovine hemoglobin variants

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Abstract

A capillary electrophoretic technique was developed to monitor patterns of hemoglobin production in young calves subjected to multiple phlebotomies. The method is similar to one previously described for the determination of human hemoglobin variants in whole blood using isoelectric focusing. After a single collection of one-half the circulating blood volume, there were obvious alterations in hemoglobin chain variants. HbA levels diminished as blood loss increased with minimum values corresponding to maximum blood loss. HbF levels did not appear to be affected. Also visible during the regenerative process were atypical overlapping peaks preceding the normal hemoglobin peaks. At the conclusion of the 18-day study, most of the electropherograms had returned to initial states. These changes were found to be a sensitive indicator of accelerated erythropoiesis in contrast to the standard technique of total hemoglobin determination by colorimetric means.

1. Introduction

Calves have become popular as animal models in artificial organ research [1–3]. Significant blood loss can be anticipated in many of the procedures to which these animals are subjected and, as a result, multiple transfusions are often required to stabilize and support the patient both during surgery and post-operatively. Consequently, donor animals specifically maintained for the purpose of providing blood are essential to this type of research.

The process of blood replacement has previously been investigated in a number of different species, however, information specific to the bovine is very limited. Subsequently, recommendations concerning optimal volumes and intervals for blood collection in cattle are not currently available.

Prior to the establishment of transfusion guidelines for the young bovine, it is essential first to document some of the major hematologic alternations which routinely occur in calves experiencing blood loss. The overwhelming presence of erythrocytes in the normal circulation makes this particular cell line an obvious candidate for monitoring this replacement process. Subtle changes detectable in circulating erythrocytes may render a predictive index, satisfactory for assessing the potential for blood production.

A fundamental aspect of erythrocyte regeneration is the synthesis of hemoglobin. Hemoglobin is the primary intracellular protein of erythrocytes, comprising approximately one third of each cell's contents. It is described as a tetramer made up of four polypeptide chains, each of

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which contains a heme group in a hydrophobic pocket [4]. This particular structure contributes to the functional capacity of the cell in the transport and exchange of gases.

Five different hemoglobin variants have been identified in normal adult cattle and, of these, types A (HbA) and B (HbB) are the most frequently encountered [5]. In addition to these types, neonatal ruminant red cells are characterized by the persistence of fetal hemoglobin (HbF), which disappears gradually and is completely replaced by adult hemoglobin types by 6 to 7 months after birth [5,6].

Previous reports of the effects of blood loss on the hemoglobin variant composition of circulating erythrocytes have indicated that this may be a viable means of verifying accelerated erythropoiesis. Studies focusing on the acute erythroid expansion in human adults recovering from an anemic state have demonstrated that, initially, there is an increased percentage of circulating reticulocytes containing HbF. Postnatal reactivation of fetal hemoglobin synthesis also occurs in patients with hematopoietic disorders, such as hematologic malignancies, several aplastic–hypoplastic syndromes, and immediately following marrow transplantation [7,8].

A novel type of hemoglobin, referred to as hemoglobin C (HbC) is produced in some ruminants subjected to severe anemia, hypoxia, or intravenous injections of erythropoietin. In sheep that have been subjected to erythropoietic stress, HbC is found to replace only the HbA component in blood with no effect on the HbB fraction. Sheep that are homozygous for HbB do not develop HbC. Similarly, sheep that are heterozygous for HbA and HbB respond to stimulated red cell production by continued production of HbB at approximately 50% of the total hemoglobin, and substitution of HbC production for the HbA fraction [9–14]. A similar study conducted in a single adult cow, heterozygous for HbA and HbB production, failed to demonstrate the appearance of a new hemoglobin type after severe blood loss anemia. The relative amount of HbA and HbB remained unaltered, despite the accelerated rate of erythropoiesis [15].

Recently, a novel method for determination of human hemoglobin variants in whole blood using capillary electrophoresis was described [16]. Using a variation of this method, the goal of this investigation was to monitor hemoglobin production in young calves subjected to a series of controlled blood collections.

2. Materials and methods

Two groups of six Holstein or Holstein-Angus calves were compared in this study. The calves ranged in age from 77 to 153 days. One group served as a control. The second group was subjected to three phlebotomies over an eight-day period, each approximately one-half of the total circulating blood volume, to create a state of artificial anemia. The animals were monitored for 18 consecutive days, including daily observation and blood sampling in addition to routine weight surveillance.

Samples of 40 μl of whole uncoagulated blood in a 1:250 dilution were prepared and analyzed for total hemoglobin content. Values were derived on an automated commercial laboratory unit (Cell-Dyn 400 Hematology Analyzer, Sequoia-Turner, Mountain View, CA) that is routinely used to process animal samples. Results were reported as grams of hemoglobin per deciliter of whole blood.

One ml of well-mixed whole anticoagulated blood was added to four ml of distilled deionized water to prepare a crude hemolysate sample. The hemolyzed sample was then vortexed to assure proper mixing. A 5-μl volume of this hemolysate was then combined with 1 ml of ampholyte solution at a pH range of 3 to 10 (Bio-Lyte ampholytes, Bio-Rad Life Sciences Group, Richmond, CA) and vortexed. A 10-μl volume of the diluted hemolysate was added to 90 μl of the same ampholyte solution for a second dilution.

All separations were performed with the BioFocus 3000 automated capillary electrophoresis system (Bio-Rad Labs, Richmond, CA). All capillaries used in this study were coated internally with a covalently-attached hydrophilic

linear polymer. Capillaries were enclosed in a cartridge format and thermostated at 20°C by liquid cooling. The distance from the detector to the capillary outlet was 4.5 cm.

Isoelectric focusing of hemoglobins were carried out using coated capillaries with 17 cm × 25 μm I.D. Capillaries were purged with distilled deionized water and 10 m*M* phosphoric acid (Bio-Rad Catholyte) between separations.

The sample-ampholyte mixtures were pressure-injected into the capillary at 100 p.s.i. (689 476 Pa) for 60 s. Focusing was carried out at 7 kV constant voltage for 5 min using 40 m*M* sodium hydroxide (Bio-Rad Catholyte) as catholyte and 20 m*M* phosphoric acid (Bio-Rad Anolyte) as anolyte. Cathodic mobilization was performed by replacing the catholyte with a proprietary zwitterionic solution (Bio-Rad Mobilizer). Mobilization voltage was 8 kV and polarity was positive to negative. Single-wavelength mode detection was at 280 nm.

Data acquisition and integration analyses were performed on an integrated personal computer system using the BioFocus (Bio-Rad Life Sciences Group) software package. Reference standards of hemoglobins A, F, S, and C were obtained from Dr. Tim Wehr of the Bio-Rad Diagnostic Group, Richmond, CA.

3. Results

As anticipated, total hemoglobin values decreased in the group of phlebotomized calves following each blood collection (see Table 1). This reduction in total hemoglobin content reflects a decline in total red cells per unit of blood as a consequence of excessive loss relative to the rate of replacement.

Differences in the mean total hemoglobin values between the two groups of calves were significant with the control group having a higher minimum value and a smaller range of variation. On the average, the experimental group had a difference in the mean of not less than 4.90 points and no greater than 5.76 points at a 95% confidence interval ($p \leq 0.05$). At the end of the 18-day observation period, total hemoglobin values for the phlebotomy group were still below those of the control animals.

Table 1
Changes in total hemoglobin values[a] during the 18-day study

Day	Control group[a]	Phlebotomy group[a,b]
1	13.9 ± 1.5	12.9 ± 1.3
2	13.5 ± 0.5	9.0 ± 1.7
3	12.6 ± 0.9	8.4 ± 1.6
4	12.4 ± 1.0	5.4 ± 0.6
5	13.3 ± 1.3	5.8 ± 0.4
6	12.9 ± 0.7	6.4 ± 0.5
7	13.1 ± 1.0	7.2 ± 0.5
8	13.2 ± 1.3	8.0 ± 0.7
9	13.5 ± 0.9	5.7 ± 1.0
10	13.5 ± 1.1	5.9 ± 0.6
11	13.2 ± 1.1	6.2 ± 0.7
12	12.6 ± 0.8	7.0 ± 0.4
13	12.6 ± 1.0	7.4 ± 0.5
14	13.1 ± 1.1	8.0 ± 1.0
15	12.9 ± 0.7	8.0 ± 0.9
16	12.5 ± 0.8	8.6 ± 0.6
17	12.8 ± 0.8	8.8 ± 0.7
18	12.5 ± 1.1	9.6 ± 1.0

Total hemoglobin values expressed as g/dl (grams per deciliter) of whole blood.
[a] Mean ± one standard deviation of total hemoglobin.
[b] Statistically significant difference ($p \leq 0.05$) when compared to the control group.

By comparing electropherograms of the samples with electropherograms generated by the same methodology using reference hemoglobin standards, the identification of the various hemoglobin types was possible based on the time and order of the various separation peaks. The sequential electropherograms for individuals in the two groups could be compared and subjective information surmised based on the obvious changes in the general appearances of the electropherograms. Due to the lack of an internal standard with which to compare peak heights and areas, it was not possible to quantitate the amount of each hemoglobin chain present (see Figs. 1 and 2).

As would be anticipated, the predominant hemoglobin type present in both groups was hemoglobin A. Four calves, which were the younger members of each group, also showed a definite hemoglobin F peak immediately prior to

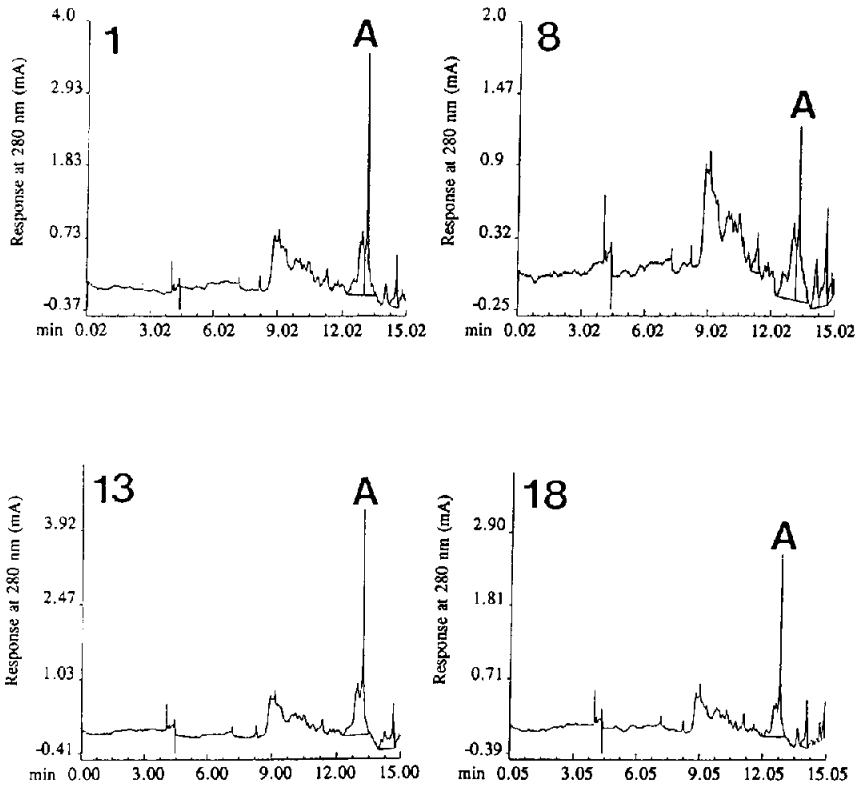

Fig. 1. Electropherograms of blood samples. A = Hemoglobin A. Numbers represent days of sampling.

the hemoglobin A peak. A definite hemoglobin C peak could not be identified in either the control group or the phlebotomized group.

The electropherograms for the control animals remained relatively stable throughout the study. Conversely, five of the six animals in the experimental group showed substantial fluctuations in hemoglobin patterns.

As blood loss increased, the hemoglobin A peak gradually diminished in height and area from initial levels. This reduction coincided with the appearance of a group of unrecognizable overlapping peaks in the region of the electropherogram preceding the hemoglobins A and F. The peak for hemoglobin C, if present, would be located in this region, however, due to the presence of other overlapping extraneous proteins, it could not be definitely identified or excluded. Minimum values for hemoglobin A during the study corresponded with the time interval associated with peak blood loss following the third phlebotomy, although changes in hemoglobin patterns were evident after the initial phlebotomy. By the end of the 18-day observation period, hemoglobin patterns for most of the experimental animals had been restored to previous, or initial, states. Actual hemoglobin F content did not appear to be altered in either the control or the experimental groups. In the experimental group, however, the relative ratio of hemoglobin F to hemoglobin A was altered as hemoglobin A levels declined.

4. Discussion

Variations in hemoglobin production as determined by capillary electrophoresis were found to be a precise indicator of accelerated erythropoiesis in the bovine. After a single collection of one-half the circulating blood volume, it could be verified that hemoglobin production patterns

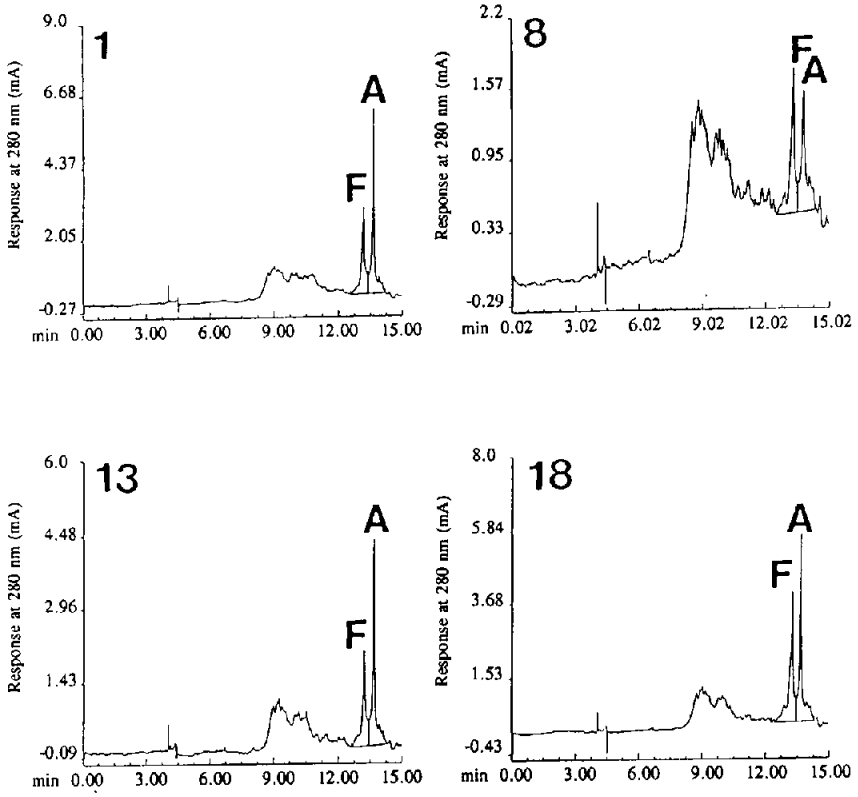

Fig. 2. Electropherograms of blood samples. A, F = Hemoglobin A and F, respectively. Numbers represent days of sampling.

were altered significantly. Likewise, altered hemoglobin production patterns were restored to normal pre-phlebotomy conditions prior to the end of the 18-day observation period.

This is in contrast to the findings in hemoglobin parameters using traditional colorimetric measurement of total hemoglobin, which has also been used as a means of anemia assessment. Fluctuations in the total hemoglobin content of blood also parallel changes in blood composition resulting from loss and replacement, however, the changes observed are neither as acute nor as sensitive as those detected by capillary electrophoresis.

The significance of these findings is that even though it is physiologically possible to collect a series of large volumes of blood from a calf, the actual composition of the collected blood is altered, even after the first collection. The effects of these changes on the functional capacity of the blood are not known, however, it can be speculated that they are not desirable. On the basis of in vitro studies in progress, it can be surmised that the alterations will most likely affect the oxygen delivering capacity and longevity of the harvested red cells [17]. Also, in the case of an animal instrumented with an artificial organ, particularly the heart, the propensity for mechanical damage to red blood cells is increased, so that any alterations in the integrity of transfused red cells are magnified.

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