Effect of ascorbic acid on storage of Greyhound erythrocytes

Jorge A. Fontes PhD
Uddyak Banerjee PhD
M. Cristina Iazbik DVM
Liliana M. Marín DVM
C. Guillermo Couto DVM
Andre F. Palmer PhD

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From the William G. Lowrie Department of Chemical and Biomolecular Engineering (Fontes, Banerjee, Palmer), and the Veterinary Medical Center (Iazbik, Couto), Department of Veterinary Clinical Sciences (Marín, Couto), Comprehensive Cancer Center (Couto), and Center for Clinical and Translational Science (Couto), College of Veterinary Medicine, The Ohio State University, Columbus, OH 43210. Dr. Fontes’ present address is DPT Laboratories Ltd, 307 E Josephine St, San Antonio, TX 78215. Dr. Couto’s present address is Couto Veterinary Consultants, 4694 Cemetery Rd, Ste 374, Hilliard, OH 43026.

Address correspondence to Dr. Palmer (palmer.351@osu.edu).

Canine blood banking provides an essential service in veterinary medicine that facilitates direct life-saving treatment for some clinical conditions and supportive treatment for other medical and surgical procedures.1–4 The use of additive solutions for the storage of canine RBCs is relatively new in veterinary medicine. In general, guidelines for transfusion and storage of canine blood components have been adapted from human transfusion medicine but have not been as thoroughly validated as guidelines for storage of human blood components. Red blood cells from both species are stored in additive solutions of specified composition to maximize posttransfusion viability and minimize RBC hemolysis.1,6–8 Currently, canine fresh whole blood can be stored in an anticoagulant CPD-adamidine solution (designated CPDA-1) for up to 35 days, or packed canine RBCs can be stored for up to 42 days in additive solutions.1,9 Canine fresh whole blood has been stored in solutions consisting of acid-citrate-dextrose10–13 or CPD11,12 with or without chemical additives10,11 as well as in a storage medium for canine blood.13 Canine RBCs have also been stored in CPDA-1,6–8 licensed RBC additive solutions (AS-1,2,3,14 and AS-3,4), and AS-3 supplemented with other factors.3

During cold (1° to 6°C) storage, human RBCs undergo glycolysis and deplete glucose that originates from the additive solution by metabolizing it to produce ATP, which forms lactic acid as a by-product.15 This biochemical pathway acidifies the additive solution and decreases the internal concentration of 2,3-DPG in RBCs16 because an acidic pH promotes depletion of 2,3-DPG by activation of bisphosphoglycerate phosphatase.17 The lack of 2,3-DPG, a key allosteric effector molecule, shifts the RBC oxygen equilibrium curve to the left, which increases the affinity of hemoglobin for O2 (lowering the P50 [ie, the P0.5 at which half of the O2 binding sites are saturated with O2]).18 Also, concentrations of ATP decrease as it is consumed to maintain an electrolyte gradient19–20 and lipid asymmetry.21–22 Red blood cells change shape during storage, changing from biconcave disks to spherocechinocytes.23–25 Vesiculation of the membrane occurs during storage whereby RBCs lose surface area relative to volume.24,26–31 Red blood cells undergo a loss

OBJECTIVE
To assess changes in biochemical and biophysical properties of canine RBCs during cold (1° to 6°C) storage in a licensed RBC additive solution (the RBC preservation solution designated AS-1) supplemented with ascorbic acid.

SAMPLE
Blood samples from 7 neutered male Greyhounds; all dogs had negative results when tested for dog erythrocyte antigen 1.1.

PROCEDURES
Blood was collected into citrate-phosphate-dextrose and stored in AS-1. Stored RBCs were supplemented with 7.1mM ascorbic acid or with saline (0.9% NaCI) solution (control samples). Several biochemical and biophysical properties of RBCs were measured, including percentage hemolysis, oxygen-hemoglobin equilibrium, and the kinetic rate constants for O2 dissociation, carbon monoxide association, and nitric oxide dioxygenation.

RESULTS
Greyhound RBCs stored in AS-1 supplemented with ascorbic acid did not have significantly decreased hemolysis, compared with results for the control samples, during the storage period.

CONCLUSIONS AND CLINICAL RELEVANCE
In this study, ascorbic acid did not reduce hemolysis during storage. Several changes in stored canine RBCs were identified as part of the hypothermic storage lesion. (Am J Vet Res 2015;76:789–800)

ABBREVIATIONS
CPD  Citrate-phosphate-dextrose
DPG  Diphosphoglycerate
GLUT1  Glucose transporter 1
GLUT4  Glucose transporter 4
HSL  Hypothermic storage lesion
MCH  Mean corpuscular hemoglobin
MCHC  Mean corpuscular hemoglobin concentration
MCV  Mean corpuscular volume
RDW  RBC distribution width
So2  Oxygen saturation of hemoglobin

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in deformability during storage\(^{16,24,32-35}\) and undergo sublethal injury, which makes them more susceptible to damage from external mechanical force, as quantified by the mechanical fragility index.\(^{36,37}\) The most susceptible or most weakened cells are lethally damaged during storage (ie, hemolysis), which increases with the progression of storage.\(^{16}\) These changes are part of the HSL.

It is hypothesized that some of the HSL is attributable to the cumulative effect of oxidative injury to RBCs during storage.\(^{38,39}\) Similar to human RBCs, canine RBCs undergo oxidative damage and hemolysis during routine storage. During hypothermic storage, biological processes that maintain RBCs in a reduced state are compromised and enzymatic protection from oxidative damage (ie, antioxidant capacity) is limited.\(^{40-44}\) This exposes stored RBCs to reactive oxygen species. Increasing concentrations of reactive oxygen species elicit hemoglobin, RBC cytoskeletal protein, and lipid oxidation.\(^{31,38,39,45,46}\) Additionally, hemoglobin autoxidation in the presence of unsaturated lipids, such as those in an RBC membrane, can result in lipid peroxidation.\(^{47}\) Spectrin oxidation during RBC storage contributes to a loss of spectrin activity (ie, binding with protein 4.1).\(^{48}\) Evidence of carbonyl group formation has identified the RBC cytoskeleton as a target of extensive oxidative damage during storage.\(^{38,39,45}\) Oxidative damage to the RBC lipid membrane and cytoskeleton destabilizes the RBC membrane, which elicits hemoglobin microvesicle release and reduces the deformability of stored RBCs.\(^{40,45,48-51}\) Oxidative damage to the RBC membrane proteins and signals clearance of RBCs after transfusion.\(^{52-54}\) Overall, the HSL negatively affects posttransfusion viability, and RBCs removed from the bloodstream after transfusion most likely consist of irreversibly damaged or damage-susceptible RBCs.\(^{55}\)

Investigators of 1 study\(^{56}\) found that supplementation of storage solution with ascorbic acid at concentrations of 5.86 and 8.78mM decreased (in a dose-dependent manner) hemolysis and mechanical fragility index in human RBCs stored in a licensed RBC additive solution. Similarly, the storage of murine RBCs with similar concentrations of ascorbic acid resulted in decreased microvesicle formation, decreased alloimmunization, and increased posttransfusion viability.\(^{57}\)

The purpose of the study reported here was to assess changes in biochemical and biophysical properties of canine RBCs during cold storage in a licensed RBC additive solution supplemented with ascorbic acid. Considering that RBCs are constantly exposed to reductants in the bloodstream, but not during ex vivo storage, we hypothesized that supplementation of additive solutions with ascorbic acid (a potent antioxidant naturally present in the blood) would create an intracellular and extracellular reducing environment for stored RBCs, which would improve RBC storage conditions by limiting the extent of oxidative damage to RBC components and result in reduced hemolysis during storage.

### Materials and Methods

**Animals**

Seven retired racing Greyhounds (all of which had negative results when tested for dog erythrocyte antigen 1.1) were selected from a pool of blood and plasma donors at the Animal Blood Bank of the Veterinary Medical Center at The Ohio State University. All dogs were neutered males to reduce interindividual variability. Blood samples were collected in accordance with an approved animal use protocol.

**RBC collection, processing, and storage**

Venous blood samples were collected from each dog and used to determine eligibility for blood donation (ie, minimum PCV for Greyhounds, 50%). For eligible donors, 450 mL of whole blood was collected into standard blood storage bags containing 63 mL of CPD (day 0). Blood bags were centrifuged at 5,895 X g for 20 minutes, and packed RBCs were manually separated from the plasma in a closed transfer system. Once these 2 fractions were separated, the plasma fraction was stored in the blood bank for future use in patients. A blood pack\(^{4}\) containing 110 mL of a licensed RBC additive solution (RBC preservation solution designated AS-1\(^3\)) was added to the packed RBCs, as recommended in transfusion guidelines.\(^1\) Each sample of packed RBCs was used to create 3 satellite bags. Two satellite bags each contained approximately 90 mL (estimated by weight) of RBCs. The other satellite bag contained approximately 200 mL (estimated by weight) of RBCs and was stored in the blood bank for use in patients. The Hct was 60% for bags containing AS-1. On day 0, a sampling port\(^5\) was aseptically attached to each of the two 90-mL satellite bags.

A stock solution of ascorbic acid (6.45 mg/mL [approx 36.6mM]) was created with saline (0.9% NaCl) solution, and pH was adjusted to 7.1 by the addition of a small amount of sodium hydroxide. A stock solution of saline solution (pH adjusted to 7.1) also was created. Stock solutions of both the ascorbic acid solution and saline solution were sterile filtered through a 0.22-μm filter before addition to satellite bags.

On day 0, the stock solution of ascorbic acid was added to each of 7 satellite bags to achieve an overall ascorbic acid concentration in the additive solution of 1.25 mg/mL (approx 7.1mM), which is approximately 125X the human plasma concentration (0.01 mg/mL [approx 0.057mM]). This concentration of ascorbic acid was chosen on the basis of studies\(^56,57\) with human and murine RBCs stored in similar concentrations of ascorbic acid, which yielded beneficial effects. An equal volume of saline solution was added to each of the remaining 7 paired satellite bags (control bags). These 2 additives were added so that the total volume
of the bags increased by 10%. Bags were stored at 1° to 6°C for 35 days (standard duration of storage).

**Blood gas analysis and CBC**

Venous blood samples obtained from each dog before blood collection on day 0 were used for baseline measurements of canine RBCs. Samples of stored canine RBCs were obtained on days 7, 21, and 35 to monitor changes in the biochemical and biophysical properties of RBCs and to assess effects attributable to the addition of ascorbic acid to the additive solution. All samples were analyzed with a blood gas and electrolyte analyzer to determine pH, PaCO₂, PaO₂, bicarbonate ion concentration, SO₂, and concentrations of sodium, potassium, and chloride ions. Samples collected in sodium EDTA were used for a CBC and analyzed with a hematology analyzer (with appropriate software settings) to determine RBC count, Hct, hemoglobin concentration, MCV, MCH, MCHC, RDW, and WBC count.

**Separation of RBCs and supernatant**

Red blood cells were removed by use of sterile technique from each satellite bag at each time point and centrifuged at 860 X g for 30 minutes at 4°C. Supernatant was aspirated manually and centrifuged again at 1,300 X g to ensure no RBCs remained. Supernatant was then harvested and used for further analysis. The remaining RBCs were washed by resuspending in chilled saline solution (volume of saline solution was 2 times the volume of the packed RBCs) followed by centrifugation of this suspension at 860 X g for 30 minutes at 4°C; the supernatant was discarded. This wash procedure was performed at least 2 times or until the supernatant became visibly clear of cell-free hemoglobin. The resulting packed RBCs were lysed in chilled 3.75 mM phosphate buffer (pH, 7.2; volume of phosphate buffer was 3 times the volume of the packed RBCs). The lysate was centrifuged at 3,716 X g for 10 minutes to remove large cellular debris; an aliquot of the centrifuged lysate was then obtained for further analysis.

**Measurement of percentage hemolysis and hemoglobin concentration**

The PCV of samples was measured after centrifugation in an Hct centrifuge at 13,700 X g for 3 minutes. The concentration of hemoglobin in both the RBC lysate and supernatant fractions was assayed via UV–visible light spectroscopy with reference to the Winterbourn equation. These concentrations were then used to calculate the percentage hemolysis in accordance with the following equation:

\[
\text{Percentage hemolysis} = \frac{(\text{Hb}_s \times \text{V}_s \times X \{1 - \text{PCV}\})}{(\text{Hb}_b \times \text{D} \times \text{V}_b \times \text{PCV}) + (\text{Hb}_s \times \text{V}_s \times X \{1 - \text{PCV}\})}
\]

where \(\text{Hb}_b\) is the hemoglobin concentration in the supernatant, \(\text{V}_s\) is the total sample volume, \(\text{PCV}\) is the PCV of the blood sample, \(\text{Hb}_l\) is the hemoglobin concentration in the lysate, and \(D\) is the dilution factor.

**Measurement of methemoglobin concentration in supernatant and RBC lysate**

The concentration of methemoglobin for both the supernatant and RBC lysate fractions was assayed via UV–visible light spectroscopy with reference to the Winterbourn equation.

**Determining \(P_{50}\) and the cooperativity coefficient**

Oxygen equilibrium curves derived for 50 µL of stored RBCs in additive solution were measured by use of dual-wavelength spectroscopy at 37°C, as described elsewhere. Briefly, samples were prepared by thoroughly mixing 50 µL of RBCs with 5 mL of buffer (pH, 7.4), 20 µL of bovine serum albumin, 10 µL of hemoglobin stabilizer, and 10 µL of antifoaming agent. Compressed air was used to saturate samples to a \(P_{O_2}\) of 147 mm Hg. The RBC samples were then deoxygenated by bubbling the solution with a compressed nitrogen stream. Absorbance of oxygenated and deoxygenated RBC samples was recorded as a function of \(P_{O_2}\), by use of dual-wavelength spectroscopy. The RBC oxygen equilibrium curves were fit to a 4-parameter Hill model with the following equation:

\[
\text{SO}_2 = \frac{(A - A_0)/(A_\infty - A_0)}{\text{P}_{O_2}^{n} + \text{P}_{50}^{n}}
\]

where \(A\) is absorbance, \(A_0\) is absorbance at 0 mm Hg, \(A_\infty\) is absorbance at full \(O_2\) saturation, and \(n\) is the cooperativity coefficient of hemoglobin.

**Measurement of 2,3-DPG concentration**

A sample obtained directly from each RBC satellite bag was used to measure the 2,3-DPG concentration with a 2,3-DPG assay kit and a UV–visible spectrophotometer as per the manufacturer’s protocol. Results were normalized on the basis of the hemoglobin concentration of each sample.

**Determining RBC gaseous ligand binding and release kinetics**

For all measurements, 1 mL of stored RBCs was sterilely removed from each satellite bag at each time point and centrifuged at 1,300 X g for 5 minutes at 4°C. Supernatant was aspirated and discarded, and the packed RBCs were washed by resuspending in chilled saline solution (volume of saline solution was 2 times the volume of the packed RBCs) followed by centrifugation of this suspension. This wash procedure was performed at least 2 times or until the supernatant became visibly clear of cell-free hemoglobin. Kinetic rate constants of these washed RBCs were measured.

All RBC gaseous ligand binding and release kinetic measurements were performed with a stopped-flow spectrophotometer as described elsewhere.
In all cases, 5 to 10 individual kinetic time course measurements were obtained and averaged. The averaged curve was then fit to a first-order exponential equation to regress the pseudo-first-order apparent rate constant for nitric oxide dioxygenation and carbon monoxide association and the zero-order rate constant for O₂ dissociation. Measuring kinetic time courses at various gaseous ligand concentrations (for carbon monoxide and nitric oxide) enabled us to plot apparent first-order rate constants against their corresponding gaseous ligand concentrations, and slope of the fitted line yielded the second-order rate constant for nitric oxide dioxygenation and carbon monoxide association.

The O₂ dissociation time courses of RBCs were obtained by rapidly mixing oxygenated RBC solutions (30 µM heme) with a deoxygenated sodium dithionite solution (1.5 mg/mL). Absorbance for the deoxygenation reaction was determined at 437.5 nm in 0.1M PBS solution (pH, 7.4) at 20°C.

Carbon monoxide association time courses of RBCs were obtained by rapidly mixing deoxygenated RBCs (30 µM heme) with a saturated carbon monoxide solution and measuring absorbance at 437.5 nm and 20°C. Both deoxygenated RBCs and carbon monoxide solutions were prepared in the presence of sodium dithionite (1.5 mg/mL); 0.1M PBS solution (pH, 7.4) was used as the reaction buffer. Apparent reaction rate constants were measured at 2 carbon monoxide concentrations (464 and 232 µM).

Nitric oxide dioxygenation time courses of RBCs were acquired by rapidly mixing oxygenated RBCs (7.5 µM heme) and appropriate dilutions of the nitric oxide stock solution and measuring changes in absorbance at 420 nm and 20°C, as described elsewhere.

**Statistical analysis**

A 2-way repeated-measures ANOVA with a Bonferroni post hoc test was used to compare differences between continuous variables in both groups at each time point for all measurements in donor RBCs. All analyses were performed with statistical software. Values of P < 0.05 were considered significant.

**Results**

**Blood gas analysis and CBC**

Results for whole blood samples obtained from Greyhounds on day 0 were summarized (Table 1). Between days 0 and 7, stored Greyhound RBCs had a decrease in PCV, Hct, and MCV and an increase in RDW (%). Table 2—Mean ± SD results of a CBC for blood samples obtained from 7 dogs and stored in cold (1° to 6°C) storage for 35 days in a licensed RBC additive solution (RBC preservation solution designated AS-1) supplemented with ascorbic acid or not supplemented (control treatment).
The concentrations of sodium, potassium, and chloride ions decreased slightly, and the concentration of bicarbonate ion decreased profoundly. There was an increase in $P_{\text{O}_2}$, $P_{\text{CO}_2}$, and $S_{\text{O}_2}$. A large decrease in pH was also quantified between days 0 and 7. The RBC count, hemoglobin concentration, MCH, RDW, and WBC count did not change between days 0 and 7.

Between days 7 and 35, there was a significant effect of storage time on stored RBCs for all CBC and blood gas measurements, except for MCH, $P_{\text{O}_2}$, $S_{\text{O}_2}$, and sodium and chloride ion concentrations (Table 2). Although there were significant temporal changes, there was no general pattern in changes for Hct, PCV, RBC count, and hemoglobin concentration. A significant increase in MCV and a significant decrease in MCHC for both treatment groups. A significant increase in RDW was detected between days 7 and 35 for both treatment groups. The WBC count was significantly affected by storage time.

Results of blood gas analysis on stored RBCs were summarized (Table 3). The pH for both treatment groups decreased significantly, reaching an acidic pH of 6.79. The $P_{\text{O}_2}$ values for samples stored for 35 days were significantly decreased (to nearly half the values for samples stored for 7 days) for both treatments. For RBCs stored in AS-1 supplemented with ascorbic acid, potassium ion concentrations increased significantly to 5.7 and 4.9mM, respectively. Bicarbonate ion concentrations decreased significantly for both treatment groups between days 7 and 35.

### Table 3—Mean ± SD results of blood gas analysis for blood samples obtained from 7 dogs and stored in cold storage for 35 days in AS-1 supplemented with ascorbic acid or not supplemented (control treatment).

| Variable | Day 7 Ascorbic Acid | | Day 7 Control | | Day 21* Ascorbic Acid | | Day 21* Control | | Day 35* Ascorbic Acid | | Day 35* Control |
|----------|---------------------|-----|--------------|-----|---------------------|-----|---------------------|-----|---------------------|-----|
| pH †     | 6.95 ± 0.02         |     | 6.94 ± 0.01  |     | 6.85 ± 0.03         |     | 6.85 ± 0.01         |     | 6.79 ± 0.04         |     |
| $P_{\text{CO}_2}$ (mm Hg) † | 45 ± 3   |     | 42 ± 2       |     | 37 ± 3              |     | 33 ± 1              |     | 24 ± 1              |     |
| $P_{\text{O}_2}$ (mm Hg)    | 258 ± 31 |     | 260 ± 24     |     | 289 ± 6             |     | 273 ± 47            |     | 292 ± 22            |     |
| $S_{\text{O}_2}$ (%)        | 99 ± 1   |     | 100 ± 1      |     | 99 ± 1              |     | 100 ± 1             |     | 99 ± 1              |     |
| Na⁺ (mM) †                  | 155 ± 1  |     | 154 ± 1      |     | 157 ± 2             |     | 154 ± 2             |     | 156 ± 2             |     |
| K⁺ (mM) †||                    | 4.2 ± 0.2 |     | 3.4 ± 0.3‡   |     | 5.3 ± 0.1           |     | 4.5 ± 0.2#          |     | 5.7 ± 0.2‡          |     |
| Cl⁻ (mM) †                   | 116 ± 1  |     | 113 ± 1†     |     | 115 ± 2             |     | 114 ± 2             |     | 115 ± 1             |     |
| HCO₃⁻ (mM) †                 | 9.1 ± 0.5 |     | 8.4 ± 0.4    |     | 6.0 ± 0.4           |     | 5.3 ± 0.1           |     | 3.4 ± 0.3‡          |     |

*Represents results for only 3 measurements. †Values differ significantly ($P < 0.05$) over time. ‡Differs significantly ($P < 0.05$) from the value for ascorbic acid on day 7. §Differs significantly ($P < 0.05$) from the value for the control group on day 7. ||Values differ significantly ($P < 0.05$) between ascorbic acid and control treatments. ¶Differs significantly ($P < 0.01$) from the value for ascorbic acid on day 7. #Differs significantly ($P < 0.01$) from the value for ascorbic acid on day 21. **Differs significantly ($P < 0.01$) from the value for ascorbic acid on day 35. ††Differs significantly ($P < 0.05$) from the value for ascorbic acid on day 7.

### Figure 1—Mean ± SD percentage hemolysis in RBCs obtained from 7 neutered male retired racing Greyhounds (all of which had negative results when tested for dog erythrocyte antigen 1.1) and stored in a licensed RBC additive solution (RBC preservation solution designated AS-1) supplemented with ascorbic acid (squares) or not supplemented with ascorbic acid (inverted triangles). Within a time period, results for the treatment groups are offset to prevent overlap of values. *†‡Values differ significantly (*$P < 0.05$; †$P = 0.001$; ‡$P < 0.001$).

### Figure 2—Mean ± SD percentage hemolysis in RBCs obtained from 7 neutered male retired racing Greyhounds (all of which had negative results when tested for dog erythrocyte antigen 1.1) and stored in a licensed RBC additive solution (RBC preservation solution designated AS-1) supplemented with ascorbic acid (squares) or not supplemented with ascorbic acid (inverted triangles). Within a time period, results for the treatment groups are offset to prevent overlap of values. *†‡Values differ significantly (*$P < 0.05$; †$P = 0.001$; ‡$P < 0.001$).

### Percentage hemolysis and methemoglobin concentrations

Percentage hemolysis increased significantly with time and reached a mean concentration of approximately 0.8% by day 35 (Figure 1). On day 35, hemolysis in 3 of the control bags and 1 ascorbic acid-treated bag surpassed the threshold of 1% set by the FDA. The methemoglobin concentration in the supernatant of stored RBCs did not change significantly between days 7 and 35 (Figure 2). Methemoglobin concentration in the supernatant remained low (approx 0.5%). Methemoglobin concentration in the RBC lysate was lower (approx 0.1%) than in the supernatant. Although methemoglobin concentrations changed significantly over time, there was no obvious pattern.

### O₂ affinity and cooperativity coefficient

The O₂ affinity as quantified by the $P_{50}$ (Figure 3) and cooperativity coefficient (Figure 4) of RBCs in...
AS-1 changed significantly over time. On day 7, the $P_{SO}$ for RBCs stored in AS-1 supplemented with ascorbic acid was 34.11 and 30.90 mm Hg, respectively; the $P_{SO}$ remained virtually unchanged through day 21. On day 35, the $P_{SO}$ was significantly lower than the $P_{SO}$ on days 7 and 21. The cooperativity coefficient changed significantly throughout storage, although a pattern was not obvious.

2,3-DPG concentration

The 2,3-DPG concentration decreased over time and significantly decreased from day 7 to 35 for both treatment groups (Figure 5). For RBCs stored in AS-1 with saline solution (control treatment), there was a significant decrease in 2,3-DPG concentration between days 7 and 21, whereas for RBCs stored in AS-1 supplemented with ascorbic acid, there was a significant decrease in 2,3-DPG concentration between days 21 and 35.
RBC gaseous ligand binding and release kinetics

The kinetic rate constant for RBC deoxygenation increased significantly for RBCs stored in the control treatment between days 7 and 35 (Figure 6). The kinetic rate constant for carbon monoxide association increased significantly for RBCs stored in AS-1 supplemented with ascorbic acid between days 7 and 35 (Figure 7). Finally, nitric oxide dioxygenation rate constants increased significantly between days 7 and 35 for both treatment groups (Figure 8). For RBCs stored in the control treatment, there was a significant increase in nitric oxide dioxygenation rate between days 7 and 21 and between days 21 and 35.

Blood gas analysis and CBC

Red blood cells stored in AS-1 supplemented with ascorbic acid were not significantly different with respect to any CBC measurements, compared with results for RBCs stored in the control treatment (Table 2). Blood gas analysis revealed a significant effect of ascorbic acid supplementation on potassium ion concentration (Table 3). For RBCs stored in AS-1 supplemented with ascorbic acid, potassium ion concentrations were significantly different on days 7, 21, and 35. Chloride ion concentrations differed between the 2 treatments on day 7, but there was no overall treatment effect of supplementation with ascorbic acid. There was a significant difference in pH on day 21 between RBCs stored in AS-1 supplemented with ascorbic acid and RBCs stored in the control treatment.

Biochemical assays

Evaluation of percentage hemolysis (Figure 1), methemoglobin concentration in the supernatant and lysate (Figure 2), O₂ affinity as quantified by the P₅₀ (Figure 3), and the cooperativity coefficient (Figure 4) revealed that supplementation of AS-1 with ascorbic acid had no effect. Similarly, the concentration of 2,3-DPG was not significantly affected by supplementation of AS-1 with ascorbic acid (Figure 5). Finally, kinetic rate constants of O₂ dissociation (Figure 6), carbon monoxide association (Figure 7), and nitric oxide dioxygenation (Figure 8) did not differ significantly between treatment groups at any time point.
Discussion

Results of CBC and blood gas analysis for initial (day 0) venous samples in the study reported here correlated well with findings in other studies. On day 7, the 2,3-DPG concentration of stored RBCs in the present study was higher than the day 0 values reported in some studies but similar to initial values reported in other studies. Previous studies on the storage of canine RBCs have involved the use of ascorbic acid to supplement additive solutions and have found no beneficial effect. Although ascorbic acid concentrations similar to those in other studies were used in the present study, sample sizes were small, and RBC storage methods differed. Although acid-citrate-dextrose and CPD were commonly used at the time of the study, they were generally replaced by CPDA-1 for storage of fresh whole blood. With component separation, additive solutions currently are used to store packed RBCs. In 1 study, 2 of 6 units of blood were modified with a solution of ascorbic acid, which changed the Hct of these 2 units but not that of the untreated (control) units. In another study, pH of the control units used for comparison varied, which may have confounded results of that study. Differences in both Hct and pH can, individually, have a significant effect on the outcome of RBC storage.

In the present study, we evaluated the biochemical and biophysical properties of RBCs over the standard 5-week storage time for canine blood. Most of these changes had not been previously reported in dogs. Overall, it was concluded that the addition of 7.1mM ascorbic acid did not reduce hemolysis, nor did it significantly affect most of the biochemical and biophysical properties of the stored RBCs.

In the study reported here, whole blood samples were obtained from canine donors on day 0, and RBCs were extracted and placed into an anticoagulant solution, allowed to cool to room temperature (approx 22°C), centrifuged and processed manually, separated from the plasma component, mixed with an additive solution, and stored at reduced temperatures for the following 7 days. During this 7-day storage period, several blood gas and CBC measurements changed; however, not all of these changes were of importance in determining changes in RBCs as a result of the HSL. Changes in PCV and Hct likely were artifacts of the manipulations of the RBCs during removal of plasma and replacement with a different volume of additive solution. The hemoglobin concentration, MCH, and RDW remained constant during the first week of storage, compared with results for day 0, because the RBCs did not lose much hemoglobin through vesiculation or hemolysis; however, MCV was reduced in Greyhound RBCs, which increased the MCHC by day 7 and indicated that the additive solution was slightly hypertonic.

Changes in concentrations of sodium, potassium, and chloride ions were likely also artifacts of changes in the volume of the liquid preservative during processing. The decrease in the concentration of bicarbonate ions was attributable to the buffering action of bicarbonate ions as well as dilution of the available bicarbonate, which was not a component of AS-1. Neutralization of free protons prevented a more profound decrease in pH. The pH still decreased sharply between blood collection on day 0 and measurement after storage for 7 days. This initial decrease in pH likely was caused by placing whole blood into CPD and subsequent storage in AS-1 (pH, 5.5), which can result in packed RBCs with a pH of approximately 7.0. The increase in P<sub>O</sub><sub>2</sub> was partially attributable to the neutralizing action of bicarbonate and the production of carbon dioxide via the pentose phosphate pathway during the first week of storage. The difference in P<sub>O</sub><sub>2</sub> between days 0 and 7 was caused by exposure of deoxygenated RBCs (50 mm Hg) from the veins to oxygen in the AS-1 (approx 145 mm Hg). During the first week of storage, additional gaseous oxygen permeates the blood bag and, at colder temperatures, has increased solubility in the additive solution, which increases P<sub>O</sub><sub>2</sub>. Leukoreduction was not performed on these samples, and the WBC count on day 0 was similar to that on day 7. Although most of these changes were likely artifacts of RBC processing, we did not obtain any measurements immediately after processing the canine RBCs and thus cannot state whether these changes were attributable to RBC processing or as a result of the HSL during cold storage for 7 days.

The biochemical and biophysical properties of stored RBCs changed significantly over the 35 days of storage. The MCV of Greyhound RBCs increased significantly between days 7 and 35, which caused a significant decrease in MCHC. This change in cell volume could have been the result of an electrolyte imbalance, which would draw water into cells and effectively reduce MCHC by diluting the hemoglobin inside RBCs. The RDW increased as a result of the volume increase in a portion of the RBC population. Mean corpuscular hemoglobin remained constant owing to low hemoglobin loss through vesiculation and hemolysis. There was a significant decrease in WBC count during storage because leukocytes degrade during cold storage.

A constant decrease in pH of stored RBCs was attributed to lactate formation and the decreased availability of bicarbonate in the RBC additive solution. When CO<sub>2</sub> is formed by the combination of hydrogen and bicarbonate ions or via the pentose phosphate pathway, it freely permeates the blood bag, which continuously reduces P<sub>O</sub><sub>2</sub> in the liquid phase of the storage system. The P<sub>O</sub><sub>2</sub> and P<sub>CO</sub><sub>2</sub> remain high because the polymer blood bag is permeable to O<sub>2</sub>, which has increased solubility at reduced temperatures. Sodium and chloride ion concentrations remain constant throughout storage because additive solutions are composed of these ions in amounts equal to those inside RBCs. Supernatant concentrations of potassium ions increased significantly by the expiration date (day 35 of storage), but these were still extremely low, compared with potassium concentrations in human RBCs.
stored until expiration,76 and were similar to those in other studies.3-7 The increases in potassium concentration likely were a result of disintegrating leukocytes (ie, decrease in WBC count), which have high intracellular potassium ion concentrations,77 and was not necessarily attributable to the release of intracellular potassium ions from RBC hemolysis or failure of the RBC ATPase pump.3-5 Although it is common for human RBCs to be leukoreduced,78,79 we did not perform leukoreduction on these canine blood samples, although it may be beneficial to recipient dogs.14

During the last weeks of storage, the O2 affinity of stored RBCs increased markedly, as indicated by a sharp decrease in PaO2. This most likely was a result of the progressive decrease in the 2,3-DPG concentration inside the RBCs.80 Transfusion of stored RBCs in humans results in rapid regeneration of the concentration of this key allosteric effector81,82; in 1 study,83 there was a 40% regeneration of 2,3-DPG concentrations by 3 hours after transfusion. Hemolysis during ex vivo storage could also cause an increase in oxygen affinity of stored blood, given that it is a mixture of intact RBCs as well as hemoglobin microvesicles and cell-free hemoglobin, both of which have a higher O2 affinity than RBCs.

Rates of O2 dissociation from oxygenated hemoglobin, carbon monoxide binding to deoxygenated hemoglobin, and nitric oxide dioxygenation of oxygenated hemoglobin increased significantly during the storage period. Nitric oxide dioxygenation rate constants more than doubled, whereas deoxygenation and carbon monoxide binding increased by 5% to 10% during storage for 35 days. Changes in O2 dissociation rates differed from those determined by our research group (unpublished results) and those of a study84 that revealed no change in these kinetic rate constants during storage of human RBCs in another licensed RBC additive solution (designated as AS-5) for 42 days. The causes of these differences are undetermined, but we speculate that they are inherent to the species from which the RBCs are obtained.

A high O2 dissociation rate constant could lead to vasoconstriction in accordance with the autoreregulation theory, which hypothesizes that excess O2 delivery to the tissues will be counteracted by a reduction in blood vessel diameter (ie, vasoconstriction) to reduce O2 transport to the surrounding tissues.85 In this scenario, stored RBCs may not deliver enough O2 because of the increased O2 affinity associated with decreased 2,3-DPG concentrations at the end of storage. The clinical outcome and relevance of these 2 changes are unclear and may only be of consequence in a recipient requiring a large transfusion volume.

An increase in the magnitude of the nitric oxide dioxygenation rate constant could lead to scavenging of endothelial-derived nitric oxide by RBC-encapsulated oxygenated hemoglobin. In the absence of a putative vasodilator such as nitric oxide, localized vasoconstriction occurs.86 However, vasoconstriction is not a likely result of this increase in nitric oxide dioxygenation rate, since there are other barriers to endothelial nitric oxide diffusion to the circulating RBCs that would decrease the rate of scavenging by RBCs.87-90 Changes in nitric oxide homeostasis will be dominated by the presence of cell-free hemoglobin and hemoglobin microvesicles in stored RBCs, both of which have been found to scavenge nitric oxide at a much higher rate than that for intact RBCs.91

Some of the biochemical and biophysical changes for the canine RBCs of the present study have been reported for human RBCs during routine storage for 42 days. Investigators of 1 study56 found that supplementation with ascorbic acid at concentrations of 5.86 and 8.78 mM decreased hemolysis and mechanical fragility index in human RBCs stored in AS-5 in a dose-dependent manner. Supplementation of ascorbic acid in stored human RBCs also significantly reduced hemolysis after storage in AS-5 for 42 and 56 days.56 The addition of ascorbic acid to the RBC additive solution may have yielded better results for those human RBCs, compared with results for the canine RBCs of the present study, because of the lack of leukoreduction for the canine packed RBCs. Metabolically active leukocytes consume glucose and expose cells to increased amounts of metabolic waste, cytokines, enzymes, and reactive oxygen species, which results in increased hemolysis and vesicle formation during storage.92-95 Also, the difference in the number of GLUT1 and GLUT4 transporters on the surface of RBCs of humans and dogs may affect the outcome.96-97 Humans, higher primates, guinea pigs, and fruit bats have greater expression of GLUT1 on the surface of RBCs, compared with expression for other species, because the aforementioned species are unable to synthesize ascorbic acid de novo. Dogs and other mammalian species that synthesize endogenous ascorbic acid have high expression of GLUT1 during the perinatal period; however, as adults, expression of GLUT1 is replaced with expression of another glucose transporter, GLUT4. The GLUT4 isoform of the glucose transporter does not transport ascorbic acid as efficiently as does GLUT1.98 Therefore, a higher dose of ascorbic acid could potentially provide a stronger driving force for ascorbic acid uptake to better preserve canine RBCs during cold storage. Increasing the flux of ascorbic acid into RBCs could provide adequate protection from oxidative damage during storage.

The study reported here had several limitations. It was focused on a single concentration of ascorbic acid, which did not provide any beneficial outcome for stored Greyhound RBCs. The use of other (ie, higher) concentrations of ascorbic acid may have yielded the effect reported in other studies.56,57 Also, ATP concentrations in venous samples were low, which indicated analytic error or a precipitous decrease between sample collection (day 0) and after storage for 7 days. Also, although Greyhounds are excellent blood donors, results for their blood may not be representative of blood banking conditions for other species of donor dogs. Studies similar to the one reported here should
be performed with a more varied canine donor population to determine effects attributable to both storage and modification of additive solutions with ascorbic acid. Similarly, leukoreduction, although recommended for the storage of RBCs, was not performed for the present study and should be included in studies with RBCs from dogs with higher WBC counts.

In the present study, Greyhound RBCs stored for 35 days in a standard preservative solution underwent specific biophysical and biochemical changes. These changes were not prevented by the addition of 7.1mM ascorbic acid to the preservative solution.

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Footnotes

a. Blood-pack unit (containing CPD and AS-1), Fenwal Inc, Lake Zurich, Ill.
b. ADSOL (AS-1), Baxter Healthcare, Deerfield, Ill.
d. VetStat electrolyte and blood gas analyzer, IDEXX Laboratories, Westbrook, Me.
e. Procyte-Dx, IDEXX Laboratories, Westbrook, Me.
f. Autocrit Ultra 3, Beckton Dickson, Franklin Lakes, NJ.
g. UV-Vis spectrophotometer, Hewlett-Packard, Palo Alto, Calif.
h. Oli Globalworks, Bogart, Ga.
k. 2,3-DPG kit, Roche Diagnostics, Indianapolis, Ind.
m. Prism, version 6, GraphPad Software Inc, La Jolla, Calif.

Optisol (AS-5), Terumo Corp, Shibuya-ku, Tokyo, Japan.

References


