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THE EFFECTS OF ANTICOAGULANT CHOICE AND SAMPLE PROCESSING TIME ON HEMATOLOGIC VALUES OF JUVENILE WHOOPING CRANES

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Abstract: Blood collected from juvenile whooping cranes (*Grus americana*) in 2007 and 2008 was divided and placed in either the anticoagulant lithium heparin (LiHep) or tri-potassium ethylenediaminetetraacetic acid (K_3EDTA) for diagnostic hematology. Thin smears were prepared from the anticoagulated blood in the field with no delay and in the laboratory after a 4-6-hour delay, and then used to determine differential and total leukocyte counts. Manual heterophil and eosinophil counts were greater in LiHep-treated samples compared to K_3EDTA samples ($P < 0.05$), but there was no difference in the total leukocyte concentration or differential leukocyte counts between anticoagulants based on blood smears prepared with no delay ($n = 15$). Marked differences were noted in relative heterophil ($P < 0.05$) and lymphocyte ($P < 0.05$) counts and total leukocyte ($P < 0.05$) concentrations of K_3EDTA-treated samples processed after the delay ($n = 7$), suggesting a negative effect on lymphocyte integrity from the anticoagulant. Microscopically, lymphocytes were more intact and easily differentiated from thrombocytes in LiHep-treated samples than K_3EDTA, but modest thrombocyte clumping in the LiHep samples was a concern. Either anticoagulant appears adequate for diagnostic hematology in juvenile whooping cranes based on this limited analysis, but blood smears should be prepared immediately under controlled conditions for best results.

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Key words: anticoagulant, ethylenediaminetetraraacetic acid, *Grus americana*, hematology, heparin, whooping crane.

Historically, the International Crane Foundation (ICF) has used di- or tripotassium ethylenediaminetetraacetic acid (K_3EDTA) as their anticoagulant of choice for hematology studies of cranes, except for *Balearica* sp., due to frequent clot formation and/or red cell hemolysis (Samour 2006). In 2000, ICF veterinary staff began routinely preparing blood smears immediately after collection in the field instead of waiting to return to the laboratory. Observations of lower relative (%) heterophil to lymphocyte ratios and markedly increased total leukocyte concentrations became common, particularly among juvenile whooping cranes (*Grus americana*). Hematology results from samples using smears made after an unavoidable delay (typically 30 min or more) were more often consistent with reference ranges from captive whooping cranes (Olsen et al. 1996, 2001), which were likely based on samples with delays in processing.

We hypothesized that whooping crane blood in contact with K_3EDTA for an extended period of time resulted in degeneration of lymphocytes, thereby falsely lowering relative lymphocyte counts and the calculated leukocyte concentrations determined by the standard indirect method (Dein et al. 1994). We also hypothesized that an alternative anticoagulant, lithium heparin (LiHep), may circumvent the potential bias from delayed blood smear preparation, allowing more flexibility when working under conditions where a delay is expected. The objectives of our study were to compare hematology results between blood samples of whooping cranes treated with either K_3EDTA or LiHep, and with blood smears prepared with either no delay or a 4-6-hour delay. Our goal was to begin to determine optimal hematology techniques for this species under a variety of conditions.

METHODS

We collected blood samples for hematological analysis from juvenile whooping cranes ($n = 49$) during pre-migration or pre-release health examinations in fall 2007 and 2008. The cranes were being conditioned for reintroduction at the Necedah National Wildlife Refuge, Necedah, Wisconsin (Langenberg et al. 2002). Within 1 minute of manual restraint, blood was collected via jugular venipuncture and divided into tubes that contained either dry LiHep (Aktiengesellschaft, Germany; 35 IU/ml sample) or liquid K_3EDTA (Tyco Healthcare Group, Mansfield, MA; 3.0 mg/ml sample). The samples were gently rocked and blood smears were made within 1-2 minutes post-collection (no delay). The smears were made in

the field as weather conditions permitted or in a controlled environment nearby by 2 experienced veterinary technicians (CK and BR). Blood smears were prepared using the cover slip to slide technique (Samour and Howlett 2008), air-dried, and placed in fixative (JorVet™ DipQuick, Jorgensen Laboratories Inc., Loveland, CO) for 10 minutes prior to storage. All samples were then transported to the laboratory on ice packs for further analysis beginning approximately 4-6 hours following sample collection. In the laboratory, another set of blood smears were made using the same methods and fixed (delayed), and then all smears were stained with DipQuick Stain and examined with a light microscope. The total granulocyte concentration (heterophils and eosinophils; H/E concentration) of each of the divided samples was calculated using the indirect eosinophil Unopette (Becton Dickinson, Franklin Lakes, NJ) and hemacytometer method (Dein et al. 1994, Olsen et al. 1996). The relative (%) leukocyte counts of each of the divided samples were determined using the no delay and delayed blood smears from each anticoagulant when available. The 2 by 2 study design yielded up to 4 total leukocyte concentrations and differential cell counts from each blood sample (LiHep-no delay, K_3EDTA-no delay, LiHep-delayed, K_3EDTA-delayed).

Due to significant variation in field conditions (high humidity) and blood smear quality noted during the study, we imposed several subjective quality assurance (QA) criteria for the inclusion of paired samples from each crane in our analysis. Specifically, blood smears were first evaluated visually for obvious water damage and poor slide making technique (spotting, streaking, non-uniform distribution of blood). Blood smears that were considered acceptable were then evaluated microscopically at 40× to determine if a consistent monolayer was present or if leukocytes were clumped along the edges of the smear. The smears were then evaluated at 500× to assess the extent of smudged cells and if cell types were distinguishable. Blood smear pairs were removed from the study if 1 or both smears demonstrated an inconsistent monolayer, leukocyte clumping, numerous smudged cells, or if the observer was unable to distinguish leukocyte cell types due to poor staining. Differential counts were performed at 1,000× on pairs of QA-rated blood smears by a single non-blinded observer (BR) to limit inter-observer bias.

The mean, standard deviation, and minimum and maximum values were calculated for each of the hematological variables [WBC ($\times 10^3$ /ul), heterophils (%), lymphocytes (%), eosinophils (%), monocytes (%), and H/E ($\times 10^3$/ul)] from blood samples with paired QA-rated smears. The data from samples with smears prepared with no delay were log transformed to minimize skew and kurtosis of the distributions. A paired sample *t*-test was used to compare the values from the 2 anticoagulant treatments for each individual (LiHep-no delay vs. K_3EDTA-no delay). The nonparametric Wilcoxon signed rank test without data transformation was used to compare values from samples processed at the 2 time points for each anticoagulant treatment per individual (LiHep-no delay vs. LiHep-delayed; K_3EDTA-no delay vs. K_3EDTA-delayed). Statistical significance was established at $P < 0.05$. Data were analyzed using Statview 5.0.1 software (SAS Institute Inc., Cary, NC).

RESULTS

The mean ± SD H/E concentration from LiHep samples ($10.3 \pm 4.3 \times 10^3$/µl, range 3.6-25.0, $n = 49$) was greater than K_3EDTA-treated samples ($9.8 \pm 4.0 \times 10^3$/µl, range 3.2-21.8) ($P < 0.05$).

Despite using a standardized technique and trained personnel, considerably fewer cranes had

Table 1. Descriptive statistics (mean ± SD and range) for hematologic values of paired whooping crane blood samples processed without delay by anticoagulant (*n* = 15).

	WBC (×103/µl)	Heterophils (%)	Lymphocytes (%)	Eosinophils (%)	Monocytes (%)
LiHep	34.8 ± 13.6 (17.4-71.7)	24.7 ± 8.4 (9.0-37.0)	64.4 ± 8.1 (54.0-84.0)	6.0 ± 3.1 (1.0-11.0)	3.3 ± 2.1 (0.0-6.0)
EDTA	33.2 ± 10.6 (17.1-50.9)	24.5 ± 7.1 (15.0-39.0)	67.0 ± 7.4 (49.0-77.0)	5.0 ± 2.4 (1.0-11.0)	3.7 ± 1.9 (1.0-9.0)

Table 2. Descriptive statistics (mean ± SD and range) for hematologic values of 7 paired EDTA- and LiHep-treated blood samples by time of blood smear preparation.

	EDTA			LiHep		
	WBC (×10³/µl)	Hets (%)	Lymphs (%)	WBC (x10³/µl)	Hets (%)	Lymphs (%)
No delay	40.6[a] ± 10.6 (20.1-50.9)	21.0[a] ± 6.3 (15.0-30.0)	70.3[a] ± 4.7 (65.0-77.0)	32.8 ± 8.4 (17.4-41.5)	25.4 ± 6.1 (19.0-34.0)	65.6 ± 6.0 (57.0-71.0)
Delayed	22.6[a] ± 10.3 (8.4-38.4)	48.2[a] ± 16.6 (23.0-68.0)	43.1[a] ± 19.1 (21.0-67.0)	31.2 ± 8.8 (19.9-41.4)	29.6 ± 7.6 (20.0-44.0)	61.9 ± 6.6 (50.0-71.0)

[a]$P < 0.05$.

paired, QA-rated, no delay blood smears available for analysis (n = 15). Samples that failed to meet QA criteria were distributed approximately equally between both anticoagulants. There were no significant statistical differences in heterophil (%), lymphocyte (%), eosinophil (%), and monocyte (%) counts, or total leukocyte concentrations between the anticoagulants from paired no delay blood smears (Table 1).

A lower number of QA-rated smears from delayed preparations were available for paired analysis with the no delay smears from the same individual crane (n = 7). Table 2 shows the total leukocyte concentrations, heterophil (%), and lymphocyte (%) counts of the paired K_3EDTA- and LiHep-treated samples by time of blood smear preparation (further analysis of eosinophils and monocytes were not conducted due to their rarity). Total leukocyte concentrations were lower, heterophil (%) counts were greater and lymphocyte (%) counts were lower in K_3EDTA-treated samples using delayed smears than the no delay smears ($P < 0.05$). No statistically significant differences were observed in the hematology measures of LiHep-treated samples regardless of processing delay.

Microscopic examination of K_3EDTA-treated blood showed rare to minimal clumping of thrombocytes. Clumps of deeply basophilic staining thrombocytes at the feathered edge of blood smears were consistently observed during examination of LiHep-treated blood. Thrombocytes within the monolayer of all smears lacked or contained minimal cytoplasm, and were consistently smaller in size than lymphocytes.

Occasionally, it was not possible to distinguish or identify lymphocytes from K_3EDTA-treated smears due to incomplete cytoplasmic staining or lack of intact cytoplasm and their similar size to thrombocytes. The cytoplasm of lymphocytes often showed tendrils at the cell edges, and the nuclear chromatin was less densely packed than expected. In smears of LiHep-treated blood, lymphocytes contained faint blue cytoplasm with distinct, intact nuclei.

Heterophils and eosinophils were challenging to differentiate in smears treated with either anticoagulant, perhaps due to staining deficiencies or changes to granular morphology (Lucas and Jamroz 1961). The color and morphology of intracytoplasmic granules, however, were typically easier to distinguish in LiHep than K_3EDTA. No basophils were observed in any smear during the course of the study.

No deficiencies were noted in the staining of granulocytes using either anticoagulant with the Unopette system. Rare clumping of leukocytes containing 2-3 stained granulocytes were observed on hemacytometers from LiHep-preserved samples. The K_3EDTA-treated samples demonstrated no such clumping.

DISCUSSION

The results of our study suggest there are minimal to no differences in most hematological values from juvenile whooping crane blood samples exposed to either LiHep or K_3EDTA when blood smears are made without delay. We found that H/E concentrations of LiHep-treated samples were greater compared to K_3EDTA-treated samples, possibly due to cell clumping observed on the hemacytometers. This difference, however, was not reflected in the total leukocyte concentrations of the samples, probably due

to the greater variation in differential leukocyte counts used in the calculations and possibly due to small sample size.

The marked differences in hematological values between K_3EDTA-treated samples with delayed processing support our hypothesis of an effect on lymphocyte integrity and bias to the subsequent calculation for total leukocyte concentration in juvenile whooping cranes. Artifacts and increased cell smudging is typically caused by prolonged exposure to anticoagulant (Campbell 1995). Our microscopic evaluations suggest that the morphology of the lymphocyte populations were negatively affected by exposure to K_3EDTA compared to LiHep and would tend to yield underestimates of lymphocyte differential counts, especially among blood which is subjected to processing delays. No negative effects on morphology of blood cells were observed between these anticoagulants in Hispaniolan parrots (*Amazona ventralis*) (Guzman et al. 2008). There did not appear to be any particular negative effect on heterophils based on our observations, only that the altered relative lymphocyte count would bias the relative heterophil count upwards. Therefore, slide preparation delay should be consistent within flock health checks, and considered when comparing normal values between studies in whooping cranes.

Immediate preparation of blood smears from juvenile whooping cranes appears to result in a more optimal diagnostic specimen and accurate test results, but must be performed under controlled conditions whenever possible. We found that despite immediate processing of samples, most of the blood smears made for this study did not achieve our QA status due to water damage from high humidity or temperature variations that affected uniformity of the smears and induced mechanical sheering artifact. The majority of smears that achieved QA status were prepared in a notably less humid shelter or vehicle. Contingencies should be made in field protocols to try and maximize slide quality through the use of chambers or other controlled enclosures.

In our experience the anticoagulant K_3EDTA normally yields adequate staining of adult whooping crane blood cells and does not tend to cause clumping of thrombocytes. In this study subjective observations of a limited number of K_3EDTA-treated samples contained lymphocytes with less cytoplasmic and nuclear definition and often appeared as smudged cells which became unrecognizable with greater delay in processing. Additionally, we found LiHep-preserved lymphocytes had maintained cellular integrity and better staining quality, and were preferred for the differential leukocyte determinations in this study. Cellular clumping in LiHep-treated samples, however, may alter the H/E concentrations determined from manual counting methods.

We chose not to test the efficacy of the staining product used in our study against brand name or in-house produced stain regimens. We suspect, however, that the current generic equivalent stain produced only marginal color differentiation in some cell populations, and that other preparations may reduce indecision problems when conducting differential counts. Further studies could include processing samples (making blood smears) in controlled environments, utilizing a different staining method, variations of anticoagulants, and a different slide making technique to maximize sample sizes.

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