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EFFECT OF HEMOLYSIS ON PLASMA PROTEIN LEVELS AND PLASMA ELECTROPHORESIS IN BIRDS

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ABSTRACT: Protein electrophoresis is recognized as a reliable diagnostic tool for birds even though results are seldom pathognomonic. Unfortunately, this technique is underused in avian medicine because many factors interfere with electrophoresis patterns; hemolysis is one of these factors and is often associated with improper specimen handling. In human laboratory medicine, hemolysis is a known interference factor that can lead to erroneous results. Published data on the influence of hemolysis on protein electrophoresis in birds is currently restricted to a single study in Psittacidae. The aim of this study was to further investigate this effect and to analyze potential interspecific differences. Blood samples were drawn from 28 Black Kites (*Milvus migrans*) and 19 Bar-headed Geese (*Anser indicus*) and separated into two aliquots. One aliquot was dipped into liquid nitrogen for 5 sec in order to cause freeze-thawing hemolysis before centrifugation. Total plasma protein concentration, plasma hemoglobin concentration, and plasma protein electrophoresis patterns were determined for both hemolyzed and nonhemolyzed samples. In both species, hemolysis resulted in falsely elevated total plasma protein concentration. In Bar-headed Geese, hemolysis caused a rise in the gamma fraction. In Black Kites, this rise involved not only the gamma fraction but also the beta fraction, stressing the potential for species-related differences. In both species, the effects of hemolysis mimicked a chronic inflammatory condition with resulting antigenic stimulation.

Key words: Bird, hemolysis, interference, protein electrophoresis.

INTRODUCTION

For human patients, serum protein electrophoresis is used extensively for diagnostic purposes (Dimpopullus, 1961; Daunizeau, 2003) and is commonly used to evaluate, diagnose, and monitor a variety of diseases and conditions, such as cancer, intestinal or kidney protein-wasting syndromes, disorders of the immune system, liver dysfunction, impaired nutrition, chronic fluid-retaining conditions, and inflammatory conditions (Le Carrer, 1998; Daunizau, 2003). The use of protein electrophoresis in veterinary medicine, especially avian medicine, is limited, but within the past 15 yr, it has been the focus of several publications and has been recognized as reliable diagnostic tool for many pathologic conditions (Lumeij, 1987; Cray and Tatum, 1998; Werner and Reavill, 1999). Unfortunately, it is under-

used due to the presence of many factors that can interfere with electrophoresis patterns; hemolysis is one of these factors. Hemolysis is recognized as an important interference factor in human laboratory medicine (Kroll and Elin, 1994); the release of hemoglobin and other intracellular components from erythrocytes can alter electrophoretic results. Release of hemoglobin usually gives a characteristic pink-to-red tinge to the plasma or the serum (Kirschbaumweg, 2001; Lippi *et al.*, 2006) and is a common phenomenon in both human and veterinary medicine (Andreasen *et al.*, 1996, 1997; Benlakehal *et al.*, 2000; Thomas, 2001). In birds, *in vivo* hemolysis is relatively rare (0.3% of the samples) and can occur as a result of acute lead toxicosis or the ingestion of crude oil by shore birds (Fry and Addiego, 1987; Fudge, 2000). *In vitro* hemolysis is more common in birds and has been

reported to affect up to 4.25% of submitted specimens (Fudge, 2000). Such hemolysis is related to improper specimen handling such as forcing blood through small needles during sampling, long storage of the blood before centrifugation, or excessive agitation when mixing (Guder, 1986).

Published information of the effect of hemolysis on serum chemistry for birds is limited to two studies (Andreasen et al., 1996, 1997). In humans and birds, hemolysis can lead to artifactual high values for plasma or serum total protein concentration measurements (Andreasen et al., 1996, 1997; Pontet, 2000; Vermer et al., 2007). There is less information for the influence of hemolysis in agarose gel protein electrophoresis. In mammals, and especially in humans, hemolysis has been shown to generate artifacts in serum electrophoresis; hemoglobin-haptoglobin complexes move to the alpha 2 fraction and free hemoglobin migrates to the beta fraction (Bossuyt et al., 1998; Benlakehal et al., 2000; Thomas, 2001). In birds, this phenomenon has only been described by Werner and Reavill (1999) in a review article, and it has been recently investigated in psittacine birds where an increase in the gamma fraction is reported (Cray et al., 2007). However, these studies were based, respectively, on isolated cases and were limited to psittacine birds. The purpose of the study reported here was to more thoroughly investigate the effects of hemolysis in plasma protein concentration measurements and electrophoresis patterns and to investigate potential inter-specific differences basing on two distant taxa.

MATERIALS AND METHODS

This study was conducted with 19 Bar-headed Geese (*Anser indicus*) held at the Clères Zoological Park (France) 49°35.910N; 001°06.498E and 28 Black Kites (*Milvus migrans*) held at the Académie de fauconnerie du Puy du Fou (France) 46°88.955N; 000°92.686E. Blood samples were performed

on the occasion of a veterinary screening protocol conducted during October and November 2007. All the birds were clinically normal. Plasma was used for all analyses. Blood samples were taken from the brachial vein for Bar-headed Geese and from the right jugular vein for Black Kites, using 21-gauge needles and 5-ml syringes. Blood (4 ml) was drawn from each bird and collected in lithium heparin tubes (Venosafe vacutainers, Terumo, Leuven, Belgium). Heparinized blood samples were immediately split into two dry tubes (Venosafe vacutainers, Terumo).

For each blood sample pair, one sample was centrifuged at 3,000×G for 5 min, whereas the other pair was dipped into liquid nitrogen for 5 sec to cause freeze-thawing hemolysis (Thomas, 2001; Lippi et al., 2006). Plasma samples were stored in cryotubes (Micronic Systems, Lelystad, Holland) at -20 C; samples were processed in 1 mo in Black Kites and 2 mo for in Bar-headed Geese. Thawed samples were rehomogenized by gentle mixing 1 hr before analysis.

Total plasma protein concentration was determined by the Biuret reaction using a Roche Integra 400 wet chemistry analyzer (Roche Integra 400©, Roche Diagnostics, Meylan, France). Readings were made at a wavelength of 552 nm. Agarose gel electrophoresis was done using a Hydrasys© semi-automated system (Sebia, Evry, France) and the Hydragel protein 15/30© set (Sebia), which is the most commonly used kit for protein electrophoresis in medical laboratories. Plasma aliquots were loaded onto the gel, and electrophoretic separation was obtained on 8 g/l agarose gels in a Tris-barbital buffer, pH 9.2, at 20 C at constant power level of 20 W, until 33 V-h had been accumulated. Once dried and colored with amidoblack, gels were read with a Preference densitometer (Sebia). This densitometer enabled us to obtain electrophoresis patterns, to define protein fractions, and to measure area under the curve (AUC) for each fraction. Albumin was identified as the largest and most anodal peak; this peak also included the prealbumin fraction. The beta peak was defined as corresponding to the fibrinogen peak. Globulins were divided into four to five fractions depending on the species. Alpha fractions were located between albumin and beta peaks, and gamma peaks were located beyond the beta peak. As described in other studies on avian protein electrophoresis, the A/G ratio was calculated by dividing the sum of prealbumin and albumin by the sum of the globulin fractions (Lumeij, 1987; Cray and Tatum, 1998). Effect of hemolysis in electrophoresis

TABLE 1. Effects of hemolysis on total protein concentration, hemoglobin concentration, and electrophoresis patterns of Bar-headed Geese (*Anser indicus*).

Parameter	Nonhemolyzed sample ^a	Hemolyzed sample ^a	Wilcoxon matched pairs Z value	Wilcoxon matched pairs P value ^b
Total protein concentration (g/l)	56.4 ± 3.6	67 ± 8.1	3.783	<0.01*
Hemoglobin concentration (g/l)	0.07 ± 0.03	1.13 ± 0.58	3.823	<0.01*
Total AUC ^c	1,707.6 ± 288.1	1,897.6 ± 328.9	2.575	0.01*
Albumin AUC ^c	950.9 ± 169.2	805.8 ± 129.3	-3.501	<0.01*
Alpha 1 AUC ^c	92.9 ± 17.3	91 ± 15.5	-0.805	0.42
Alpha 2 AUC ^c	243.5 ± 63.2	236.3 ± 58	-1.730	0.08
Beta AUC ^c	331.5 ± 60.8	344.4 ± 72.6	0.926	0.36
Gamma AUC ^c	88.8 ± 22.9	420.1 ± 199.6	3.823	<0.01*
A/G	1.26 ± 0.14	0.8 ± 0.25	-3.823	<0.01*

^a Mean ± SD.^b Asterisk (*) indicates significant differences between groups (Wilcoxon's test: $P \leq 0.05$).^c AUC = area under the curve.

patterns was investigated by comparing AUCs for each fraction rather than protein concentration values, because in hemolyzed samples total protein concentrations are often erroneous (Andreasen et al., 1996, 1997).

Hemoglobin concentration was estimated by the oxyhemoglobin method, as described by Howlett (2000); however, we used 40 µl of plasma instead of 20 µl of blood. Plasma were diluted in 5 ml of a 0.04% ammonia solution and placed on a roller mixer for 3 min. Samples were then transferred in spectrophotometric cuvettes for immediate reading. Absorbances were read at 540 nm with a Helios Delta spectrophotometer (Thermo Electron Corporation, Courtaboeuf, France). Before processing samples, the spectrophotometer was set to zero using the dilution solution alone as a blank. Machine readings were then directly converted into grams per liter referring to a calibration graph established with a commercially available hemoglobin standard (Coulter 5 C control, Beckman Coulter Inc., Roissy, France). Migration distances were measured directly on the gels. These measures were made from the edge of the gels with a 6" dial calliper square (General, Montreal, Canada). Systat 7.0 software (SPSS Inc., Chicago, Illinois, USA, 1997) was used for all analyses. Considering the small size of the population studied, Wilcoxon's signed rank test, Mann-Whitney *U*-test, and Spearman rank correlation were used.

RESULTS

In both species, hemolysis resulted in a significant increase in plasma hemoglobin

(Tables 1 and 2); mean hemoglobin concentrations increased from 0.07 g/l to 1.13 g/l in Bar-headed Geese and from 0.08 g/l to 0.5 g/l in Black Kites. In both species, hemolysis also resulted in a significant increase in total protein concentration and AUC. Total protein concentration seemed to be correlated to plasma hemoglobin concentration (Spearman's test: $n=19$, $R_s=0.859$, $P<0.01$ [Bar-headed geese]; $n=28$, $R_s=0.486$, $P<0.01$ [Black Kites]).

Bar-headed Geese electrophoresis patterns had five fractions (Fig. 1A), and six fractions were seen with Black Kites (Fig. 1B). Black Kites had a high alpha 1 peak and two gamma peaks.

In both species, freeze-thawing hemolysis resulted in a significant increase in total AUC (Tables 1 and 2). Main changes related to hemolysis occurred in the albumin and gamma fractions. The hemolyzed samples had significant lower albumin fraction AUC in both species. Changes in these fractions represented a 17% average decrease in Bar-headed Geese and an 8% average decrease in Black Kites. In both species, hemolyzed samples had a significantly higher gamma fraction AUC. Changes in this fraction represented a 441% average increase of the gamma fraction in Bar-headed Geese and a 235% average increase in the gamma 1 fraction

TABLE 2. Effects of hemolysis on total protein concentration, hemoglobin concentration, and electrophoresis patterns of Black Kites (*Milvus migrans*).

Parameter	Nonhemolyzed sample ^a	Hemolyzed sample ^a	Wilcoxon matched pairs Z value	Wilcoxon matched pairs P value ^b
Total protein concentration (g/l)	35.5 ± 3.5	37.7 ± 3.7	3.496	<0.01*
Hemoglobin concentration (g/l)	0.08 ± 0.05	0.5 ± 0.42	4.623	<0.01*
Total AUC ^c	1,352.5 ± 84.9	1419.7 ± 99.1	3.142	0.02*
Albumin AUC ^c	604.3 ± 33.6	557.2 ± 37.3	-3.803	<0.01*
Alpha 1 AUC ^c	450 ± 52	423.2 ± 43.7	-4.076	<0.01*
Alpha 2 AUC ^c	27.4 ± 5.5	30 ± 5.3	1.526	0.13
Beta AUC ^c	171 ± 40	222.2 ± 37.4	4.463	<0.01*
Gamma 1 AUC ^c	26.7 ± 8.7	89.9 ± 51.1	4.623	<0.01*
Gamma 2 AUC ^c	69.8 ± 19	93.5 ± 19.4	4.623	<0.01*
A/G	0.81 ± 0.09	0.65 ± 0.09	-4.623	<0.01

^a Mean ± SD.
^b Asterisk (*) indicates significant differences between groups (Wilcoxon's test: $P \leq 0.05$).
^c AUC = area under the curve.

in Black Kites. In addition, these values seemed to be very well correlated to hemoglobin concentration (Spearman's test: $n=19$, $R_s=0.900$, $P<0.01$; $n=28$, $R_s=0.561$, $P<0.01$).

Hemolysis resulted in a significant decrease in the A/G ratio in the both species tested. In Bar-headed Geese, hemolysis did not significantly impact any of the fractions except albumin and gamma. In Black Kites, hemolyzed samples had a significantly lower alpha 1 fraction AUC and significantly higher beta and the gamma 2 fraction AUCs. Changes in these fractions represented an 8% average decrease in the alpha 1 fraction, a 30% increase in the beta fraction, and a 32% average increase in the gamma 2 fraction. In addition, the alpha 1 fraction was shown to be negatively correlated to hemoglobin concentration (Spearman's test: $n=28$, $R^2=-0.588$, $P<0.01$), whereas beta fraction seemed to be positively correlated to hemoglobin concentration (Spearman's test: $n=28$, $R^2=0.412$, $P<0.05$).

On gels, hemoglobin migrated a significantly longer distance for Black Kite rather than Bar-headed Goose samples (Mann-Whitney U -test: $U=474$, $P=0$). In addition, the band corresponding to the beta peak seemed to migrate over a significantly shorter distance in Black Kite

than in Bar-headed Goose (Mann-Whitney U -test: $U=0$, $P=0$). This resulted in a significantly shorter distance between beta peak and hemoglobin peak in Black Kite than in geese (Mann-Whitney U -test: $U=0$, $P \leq 0.01$).

DISCUSSION

This study demonstrates that hemolysis is an important interference factor affecting the measurement of total plasma protein concentration and plasma protein electrophoresis in birds and that these effects can be species specific. Hemolysis can increase observed total protein concentrations and can influence hemoglobin migration patterns on electrophoresis. In Bar-headed Geese, hemoglobin migrates to the gamma fraction. In Black Kites, hemolysis results in an increase in beta, gamma 1, and gamma 2 fractions. This increase seems to primarily concern the gamma 1 fraction, as suggested by its high average increase. In Black Kites, hemoglobin may therefore mainly migrate to the gamma1 fraction, the more immediate proximity between hemoglobin and beta peaks being responsible for the significant increase in beta fraction. In both species, these changes may mimic a chronic inflammatory condition with a resulting

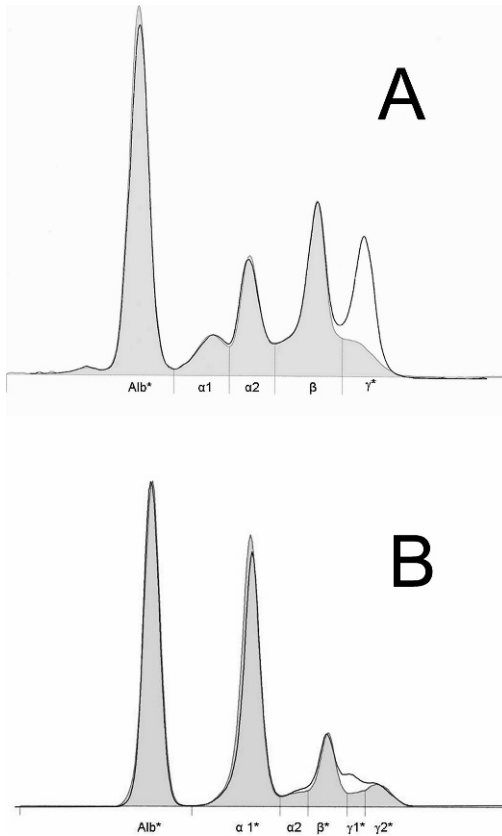


FIGURE 1. (A) Examples of plasma electrophoresis patterns of a Bar-headed Goose (*Anser indicus*). Patterns from the nonhemolyzed sample (gray curve) and the hemolyzed sample of the same bird (black curve) were superimposed. Asterisks represent significant differences between hemolyzed and non-hemolyzed samples (Wilcoxon's test: $P \leq 0.05$). (B) Examples of plasma electrophoresis patterns of a Black Kite (*Milvus migrans*). Patterns from the nonhemolyzed sample (gray curve) and the hemolyzed sample of the same bird (black curve) were superimposed. Asterisks represent significant differences between hemolyzed and nonhemolyzed samples (Wilcoxon's test: $P \leq 0.05$).

antigenic stimulation (Kaneko, 1989; Werner and Reavill, 1999). Hemolysis interference may therefore mislead the practitioner. Further studies should investigate this phenomenon in other species and try to define a threshold value for hemoglobin concentration in birds beyond which samples should be preferably rejected. In this study, measuring packed cell volume before and after hemolysis could have

helped us to assess the proportion of lysed cells after the freezing of the samples.

In a hemolyzed sample, constituents released in the plasma or serum from broken blood cells can interfere with laboratory results in three ways: 1) Addition interference: This interference applies to analytes in which intracellular concentration is far more important than plasma concentration. Leakage of these intracellular components leads to increased concentration in plasma or serum (Kirschbaumweg, 2001; Lippi et al., 2006). 2) Chemical interference: Constituents released from blood cells can interfere with chemical reactions used to analyze plasma or serum components (Kirschbaumweg, 2001; Lippi et al., 2006). 3) Optical interference: Hemoglobin strongly absorbs light at 540 nm. Hemolysis therefore increases absorption in this wavelength range and causes an apparent increase in the concentration of analytes measured in this range. In wet reagent analyzers, endpoint assays read between 505 nm and 570 nm have been demonstrated to be subjected to interference by hemoglobin (Dorner et al., 1983; Kirschbaumweg, 2001; Lippi et al., 2006).

Total protein concentration measurement may therefore be prone to both addition and optical interferences, because hemoglobin itself is a protein (Pontet, 2000), and the Biuret reaction used to carry on this measurement was based on an endpoint assay read at 553 nm. This phenomenon has been quite well documented in birds (Andreasen et al., 1996, 1997). Hemolysis is described to induce artifactually higher total protein values (Lippi et al., 2006), even if this analysis is not extremely sensitive to interference by hemoglobin (Andreasen et al., 1996, 1997). However, effects of this interference can vary from one chemical analyzer to another (Andreasen et al., 1996, 1997; Meinkoth and Allison, 2007). In our study, total protein concentration was measured with a Biuret blanked method, which was said not to be signif-

icantly affected by sample hemolysis as long as hemoglobin concentration was under 5 g/l (Roche Diagnostics, 2006. Cobas Integra 400 user's manual 2006-10 V1 FR, 3 pp.). Nevertheless, in both species, results from plasmas coming from frozen-thawed blood samples indicated increased amounts of total proteins. This may be mainly related to addition interference, because optical interference may have been reduced by the blanking procedure. This may explain the good correlation between hemoglobin and total protein concentrations.

As demonstrated in this study, electrophoresis patterns of hemolyzed bird's sample mainly show a rise in the gamma fraction. These changes differ from those observed in mammals in which hemoglobin-haptoglobin complexes move to the alpha 2 fraction and free hemoglobin migrates to the beta 1 fraction (Bossuyt et al., 1998; Benlakehal et al., 2000; Thomas, 2001). In mammals, moderate hemolysis levels first lead to increases in the alpha 2 fraction, whereas the beta fraction starts to increase through heavier hemolysis as soon as haptoglobin binding capacity is overtaken (Benlakehal et al., 2000). Haptoglobin is a protein that binds hemoglobin with high affinity to inhibit its strong oxidative activity (Gutteridge, 1987) and to avoid it to pass through the glomeruli, which may lead to renal failure (Lim et al., 2000). However, a recent publication shows that haptoglobin does not exist in chickens and may be replaced by another hemoglobin-binding protein called PIT 54 in the Neognathae subclass. PIT 54 has been identified to be a soluble member of the family of scavenger receptor cysteine-rich proteins and seems to exist only in birds, based on the currently available genomic data (Wicher and Fries, 2006). This protein is completely different from haptoglobin, which may partly explain that no rise in alpha fraction was observed in electrophoresis of hemolyzed samples in this study.

The significant decreases observed in

albumin fraction in Bar-headed Geese and in albumin and alpha 1 fractions in Black Kites may be related to a dilution phenomenon. Indeed, blood cell lysis leads to intracellular fluid release in the plasma (Lippi et al., 2006). However, these variations may not be clinically relevant, because they are lower than interindividual variability in both species (Tables 1 and 2).

As for any biochemical parameter, good quality of the sample warrants reliability of the results, and care must be taken during both sample collection and processing. In human medicine, *in vitro* hemolysis has been documented to occur with mechanical destruction, freezing, hyperosmotic shock, detergents, exhaustion of glucose in the sample, or increased fragility due to inherited diseases (Guder, 1986). The practitioner should therefore avoid rapidly forcing blood through small needles, long storage of the blood before centrifugation or excessive agitation when mixing. Furthermore, as suggested in other studies, plasma should be used preferably to serum, for protein electrophoresis in birds (Lumeij, 1987; Cray and Tatum, 1998; Hochleithner, 1994; Werner and Reavill, 1999). The inevitable hemolysis occurring during the processing of a serum can be a significant problem, because the fluid component of the blood is in contact for a longer period than for plasma (2 hr according to the National Committee for Clinical Laboratory Standards) (Fudge, 2000; Hrubec et al., 2002). The presence of fibrinogen in the plasma is an advantage rather than an inconvenience in birds, because the fibrinogen has been demonstrated to be a very good indicator of inflammation in birds (Hawkey and Hart, 1988). In avian medicine, electrophoresis is mainly used in the diagnosis of inflammatory condition. The presence of a fibrinogen peak in avian electrophoregrams may therefore not be considered to be an issue, as is the case in human medicine in which it is currently used for the diagnosis of monoclonal gammopathies (Le Carrer, 1998; Daunizeau, 2003).

In case of sample hemolysis, another blood sample should preferably be performed (Kirschbaumweg, 2001; Martinez-Subiela et al., 2002; Meinkoth and Allison, 2007). As shown in this study, effects of hemolysis not only depend on the analyzer and the method used but also on the species. It is therefore difficult to suggest hard and fast guidelines about sample management. Samples should always be collected in transparent containers so that the practitioner could estimate sample hemolysis after centrifugation. Hemolyzed samples preferably should be discarded if hemolysis is visible as a pink-to-red hue or if the serum has an extracellular concentration of hemoglobin above 0.6 g/l (9.3 M; Lippi et al., 2006); results from the present study indicate that significant changes can be induced at or above this concentration. If impossible to obtain another sample, results should be reported with a warning about possible hemolysis artifacts (e.g., hemolyzed sample: total protein concentration and gamma fraction may be overestimated).

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