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The effect of EDTA, heparin and storage on the erythrocyte osmotic fragility, plasma osmolality and haematocrit of adult ostriches (*Struthio camelus*)

Benford Mafuvadze[1], and Kennedy Honey Erlwanger[2]*

[1]*Preclinical Department, Faculty of Veterinary Science, University of Zimbabwe, Mt Pleasant Harare, Zimbabwe*

[2]*School of Physiology, Faculty of Medicine, University of the Witwatersrand, Johannesburg, South Africa*

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ABSTRACT

To investigate the effects of two widely used anticoagulants and storage on the *in vitro* osmotic fragility of erythrocytes, haematocrit (Hct) and plasma osmolality of ostriches, blood was collected by venipuncture from 8 birds (90-110 kg) into vacutainer tubes containing either EDTA (k_3) or lithium heparin. The osmotic fragility of the erythrocytes was determined by measuring the release of haemoglobin from blood added to tubes containing serially diluted phosphate buffered saline (PBS, pH 7.4). The Hct was determined by microcentrifugation and osmolality with a cryoscopic osmometer. Blood samples were analysed at 0.5 h, 6 h and 12 h after collection. EDTA increased the erythrocyte osmotic fragility compared to heparin ($P<0.01$). The initial haemolysis (>5%) occurred at between 0.50% and 0.55% PBS in heparin. In 0.85% PBS the haemolysis of samples collected in EDTA was greater than 30%, whereas in heparin it was <5%. The mean corpuscular fragility was between 0.35 and 0.45% PBS in heparin and 0.85% PBS in EDTA. Maximum haemolysis occurred in 0.35% PBS with EDTA and in 0.20% PBS with heparin. Hct was not significantly affected by storage ($P>0.05$). However, Hct values were significantly lower in heparin compared to EDTA ($P<0.05$). For EDTA and heparin, the osmolalities obtained 30 minutes after collection were significantly lower ($P<0.05$) than after 6 hours and 12 hours. However, there was no significant difference in the osmolality of the plasma 6 hours after collection compared to 12 hours. Plasma in EDTA had a significantly higher ($P<0.05$) osmolality than that in heparin.

Key words: ostrich, anticoagulants, erythrocyte fragility, haematocrit, osmolality

Introduction

Ostrich farming is a major agro-industry. The birds are mainly reared for meat, leather and feathers. Due to the semi-intensive to intensive nature of ostrich farming it

*Contact address:

Dr. Kennedy H. Erlwanger, BVSe, MSc, PhD, School of Physiology, Faculty of Medicine, University of the Witwatersrand, 7 York Rd, Parktown 2193, Johannesburg, South Africa, Phone: +27 11 7172 154; Fax: + 27 11 6432 765; E-mail: kennedy.erlwanger@wits.ac.za

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is important to regularly monitor flocks for disease and micronutrient nutritional status. Haematology and blood biochemistry are routinely used in veterinary medicine to evaluate the health status of animals and poultry. Collection of samples from abattoirs is a useful tool for routine monitoring of the health status of animals. In collecting blood samples, ethylene diamine tetracetic acid (EDTA) is the anticoagulant of choice for haematology in mammals, whereas for ostriches, heparin is the most accepted anticoagulant for biochemistry tests (PERELMAN, 1999). The mean corpuscular fragility of erythrocytes may reflect phylogenetic characteristics as well as an animal's ability to respond to osmotic challenges associated with cyclic dehydration and rehydration (BUFFENSTEIN et al., 2001). The osmotic fragility of erythrocytes is a measure of their overall response to osmotic pressure and is influenced by several extrinsic and intrinsic factors. Extrinsic factors such as pH, temperature, osmolality and blood storage affect the osmotic fragility of erythrocytes (DACIE and LEWIS, 1995; LEWIS and FERGUSON, 1966; OYEWALE et al., 1991; OYEWALE, 1994). Placing duck erythrocytes in a hypotonic medium stimulates a chloride dependant potassium transport, and placing the duck cells in a hypertonic medium stimulates an electrically neutral co-transport of sodium, potassium and chloride (HAAS and McMANUS, 1985). These transport systems are thought to have a role in cell volume regulation. Unlike ducks that thrive in an environment with lots of water, the ostrich is more adapted to arid regions. Thus, the transporters regulating cell volume may respond differently to osmotic stresses. The haematocrit in ostriches has been observed to be well regulated and that even dehydrated birds show no or little haemoconcentration (BROWN and JONES, 1996).

It is usually not feasible to analyse blood samples collected in the field within minutes of collection and therefore several hours may elapse between collection of the samples and analysis. Storage affects the plasma biochemistry in the ostrich (VERSTAPPEN et al., 2002) whereas EDTA and sodium heparin may change the levels of calcium and sodium in the serum (PERELMAN, 1999) the effects of which on osmotic fragility of the erythrocytes has not been reported.

We therefore investigated the effects of two anticoagulants (Heparin and EDTA) that are commonly used in veterinary medicine, and time of exposure to the anticoagulants, on the fragility of erythrocytes, haematocrit and osmolality.

Materials and methods

Eight ostriches weighing between 90-110 kg were used in the study. Blood (4.5 mL) was collected by venipuncture (20G vacutainer needles) from the jugular vein into vacutainer tubes containing either EDTA (k_3) or lithium heparin and stored at an ambient room temperature of 28 °C. The red blood cell fragility, haematocrit and osmolality were determined 30 minutes, 6 hours and 12 hours after collection.

Determination of osmotic fragility. Osmotic fragility of the erythrocytes was determined by adding 20 microlitres of blood to tubes containing 5 mL of phosphate buffered saline (pH 7.4) of serial concentrations ranging from 0 - 0.85% saline. The mixtures were allowed to stand for 60 minutes at room temperature (24 °C) and then centrifuged (Heraeus Omnifuge, Germany) at 1580 g for 5 minutes. The supernatant was decanted and its haemoglobin was determined spectrophotometrically (LKB ultrospec II, LKB Biochrom Ltd, England) at 540 nm using distilled water as a blank. The percentage of haemolysis in each concentration of buffered saline was calculated assuming 100% haemolysis in the concentration with the highest absorbance.

Determination of haematocrit. The haematocrit (Hct) of the blood samples from the two groups was determined by microcentrifugation.

Determination of osmolality. A cryoscopic osmometer (Osmomat 030) was used to determine the osmolality of the plasma samples.

All data are expressed as mean ± SD. One-way analysis of variance followed by Tukey-Kramer multiple comparisons test was used. The study was approved by the University of Zimbabwe Research Committee.

Results

Erythrocyte fragility. Figure 1 shows the effect of anticoagulants and storage on the fragility of the erythrocytes from the ostriches. Blood collected in EDTA had a significantly ($P<0.01$) increased osmotic fragility compared to blood collected in Heparin. In 0.85% phosphate buffered saline (PBS) the samples collected in EDTA had greater than 30% haemolysis whilst those in heparin had less than 5% haemolysis. The initial haemolysis (>5%) occurred at between 0.50 and 0.55% PBS for the erythrocytes collected in heparin.

Table 1. Effect of anticoagulants and storage time on the haematocrit of erythrocytes from ostriches (n=8)

	Hct of blood collected in EDTA		Hct of blood collected inHeparin	
Time after collection	Range (%)	Mean ± SD (%)	Range (%)	Mean ± SD (%)
30 minutes	46 - 57	52.6 ± 4.7[a]	40 - 52	45.3 ± 4.1[b]
6 hours	47 - 59	53.6 ± 5.1[a]	44 - 51	47.3 ± 3.0[b]
12 hours	45 - 55	52.0 ± 4.1[a]	43 - 53	47.8 ± 3.4[b]

[a.b] Different superscripts indicate significant difference ($P<0.05$) of means same row

Table 2. Effect of anticoagulants and time of storage on the osmolality of plasma from adult ostriches (n = 8)

	Osmolality of plasma in EDTA		Osmolality of plasma in Heparin	
Time after collection	Range (mosm.kg^{-1})	Mean ± SD (mosm.kg^{-1})	Range (mosm.kg^{-1})	Mean ± SD (mosm.kg^{-1})
30 minutes	324 - 350	336.6 ± 8.7^{a}	321 - 343	329.5 ± 8.1^{a}
6 hours	345 - 372	364.3 ± 11.47^{a}	340 - 358	348 ± 8.5^{b}
12 hours	347 - 385	361.6 ± 13.8^{a}	330 - 354	342.8 ± 9.8^{b}

$^{a.b}$ Different superscripts indicate significant difference (P<0.05) of means same row

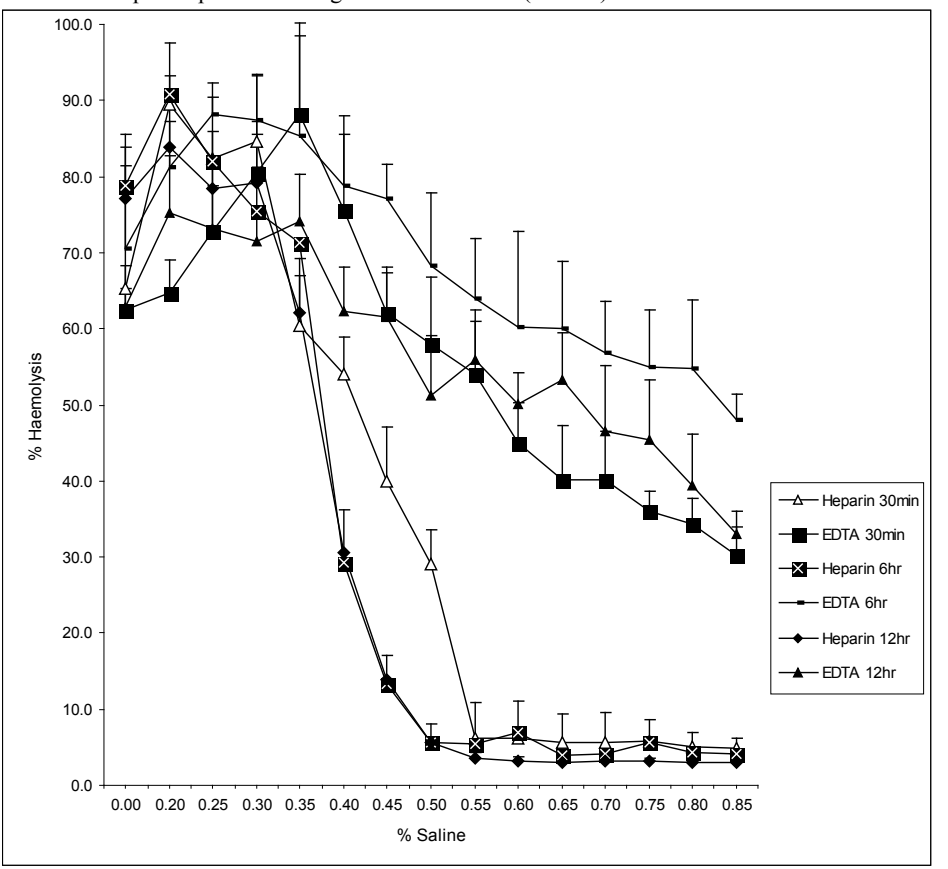

Fig. 1. Effect of anticoagulants and storage on *in vitro* osmotic fragility of erythrocytes from ostriches (n = 8)

The mean corpuscular fragility (50% haemolysis (SUESS et al., 1948)) of erythrocytes occurred in saline concentrations of between 0.35 and 0.45% for the samples collected in heparin, whereas this occurred in the 0.85% saline for the samples collected in EDTA and tested 6 hours after collection, and between 0.55% and 0.70% saline for the samples collected in EDTA and tested after 30 minutes and 12 hours of storage.

Maximum haemolysis occurred at 0.35% Saline for the samples collected in EDTA, whereas for the samples collected in Heparin it occurred in 0.20% saline.

Haematocrit. For the same anticoagulant, storage did not have a significant effect on the Hct values ($P>0.05$). However, the Hct values obtained with heparin as an anticoagulant were significantly lower ($P<0.05$) than those for EDTA (Table 1).

Plasma osmolality. For EDTA and heparin, the osmolalities obtained after 30 minutes were significantly lower ($P<0.05$) than after 6 hours and 12 hours (Table 2). However, there was no significant difference in the osmolality of the plasma 6 hours after collection compared to 12 hours. The samples collected in EDTA had a significantly higher ($P<0.05$) osmolality than those collected in heparin.

Discussion

This study has shown that both storage and type of anticoagulant used affect the fragility of ostrich erythrocytes. Although storage did not affect the haematocrit, it increased the osmolality of the plasma.

In previous studies on peafowl, pigeons, and ducks, using EDTA as the anticoagulant, initial haemolysis at room temperature was observed at 0.5% saline, whereas in 0.85% saline the haemolysis was less than 10% (OYEWALE, 1994; OYEWALE et al., 1991) which is comparable to our findings with heparinised ostrich blood. However, with EDTA we have observed that at 0.85% saline the haemolysis was greater than 30%, suggesting a possible difference in the transport systems regulating cell volume in the ostrich erythrocytes compared to the other birds. In the ostrich erythrocyte fragility seems to be affected either directly by the EDTA or indirectly by the chelation of calcium (process by which EDTA acts as an anticoagulant). The precise role of EDTA in increasing ostrich erythrocyte fragility needs further investigation. In duck erythrocytes, Na-K-2Cl cotransport activity increases tenfold in response to osmotic cell shrinkage and noradrenaline (LYTLE, 1997; LYTLE, 1998). Osmotic shrinkage of duck erythrocytes induces a net uptake of solute via Na-K-2Cl co-transport in a process that is regulated by a volume sensitive protein kinase (LYTLE, 1998). Beta adrenergic catecholamines stimulate ion transport in duck erythrocytes via cAMP (HAAS and McMANUS, 1985; LYTLE, 1998). In the pre-slaughter ostriches used in our study, the stress of the unfamiliar abattoir environment could have raised the hormones and could have affected erythrocyte membrane transport processes associated with electrolyte and water homeostasis in the erythrocytes making them

more fragile when placed in hypotonic solutions. These transport systems could have been activated in the ostriches so that when the blood was placed in the hypotonic test solutions the intracellular ion concentration could have resulted in rapid cell swelling and consequent lysis.

The maximum fragility of the ostrich erythrocytes occurring between 0.20 - 0.4% is in agreement with earlier findings for birds (LEWIS and FERGUSON, 1966; STURKIE and GRIMINGER, 1986). Mammals show maximal fragility in distilled water, whereas with ostriches there is an apparent increase in resistance to very low concentrations of PBS. This phenomenon has been attributed to the retention of haemoglobin by the cell membranes of the erythrocytes which form watery ghosts after lysis in very hypotonic solutions (PONDER, 1948). Thus, when lysis is determined indirectly by absorbance, due to the haemoglobin retention by the "ghosts", there is an apparently lower concentration of haemoglobin in the hypotonic solution, which may be erroneously interpreted as reduced fragility (as seen in Figure 1. where the% haemolysis appears to decrease with PBS concentrations less than 0.4%).

Young ostriches have lower haematocrit values than older ones (BROWN and JONES, 1996; PALOMENQUE et al., 1991; RAUKAR and SIMPRAGA, 2005). The haematocrit of young ostriches has been reported to vary between 35% and 39% while that of adult ostriches has been reported as varying between 40% and 54% in studies in which heparin (BROWN and JONES, 1996) and sodium citrate (OLOWOOKORUN and MAKINDE, 1998) were used as anticoagulants; this is in agreement with our findings using heparin as an anticoagulant. The values we obtained using EDTA were, however, significantly higher than those for heparin, suggesting that either there was lysis or cell shrinkage of the erythrocytes in the presence of heparin, or there was cell swelling (causing a relative increase in the mean corpuscular volume) in the presence of EDTA. The significantly lower haemolysis of blood collected in heparin observed in the isotonic saline negates the argument in favour of heparin causing increased cell lysis. The results thus obtained suggest that the increased haematocrit could be due to increase in mean corpuscular volume (MCV) due to swelling. However, MCV was not investigated in this study.

The haematocrit and plasma osmolality in ostriches is so well regulated that even dehydrated birds show no or little haemoconcentration (BROWN and JONES, 1996). In chicks the haematocrit was reported as 35.1%, rising to 36.5% after five days of dehydration (GRAY et al., 1988). LOUW et al. (1969) reported no changes in osmolality in adult ostriches after 11 days of dehydration, unlike in chickens were osmolality was found to increase during extended periods of dehydration.

It is also important to note that the erythrocytes were tested in PBS at room temperature (24 °C) which is almost two-thirds the normal core body temperature of ostriches and could also have had an effect on the erythrocyte membrane transport processes.

Further studies are recommended to investigate the effect of EDTA and calcium on the erythrocyte membrane transport systems of ostriches and also the effect of hormones on these systems. Nearly all routine haematologic and biochemical investigations can be performed with blood placed in lithium heparin (VERSTAPPEN et al., 2002). Our results confirm the recommendation that Lithium heparin rather than EDTA should be used as the anticoagulant of choice when dealing with ostrich blood.

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SAŽETAK

Istražen je učinak dvaju naširoko rabljenih antikoagulanata i pohrana eritrocita na njihovu *in vitro* osmotsku krhkost, hematokrit i osmolalnost plazme u nojeva. Krv je bila uzeta iz vene osam nojeva (90-110 kg) u epruvetu s vakuumom koja je sadržavala ili EDTA (k3) ili litij-heparin. Osmotska krhkost eritrocita bila je određena mjerenjem oslobođenoga hemoglobina iz krvi dodane u epruvete sa serijski razrijeđenom fosfat-puferiranom fiziološkom otopinom (PBS, pH 7,4). Hematokrit je bio određen mikrocentrifugiranjem, a osmolalnost krioskopskim osmometrom. Uzorci krvi bili su analizirani 0,5, 6 i 12 sati nakon uzimanja. EDTA je povećala osmotsku krhkost eritrocita u usporedbi s heparinom ($P<0,01$). Početna hemoliza (>5%) pojavila se između 0,50 i 0,55% PBS-a u heparinu. U 0,85% PBS-u hemoliza uzoraka uzetih u EDTA bila je veća od 30%, dok je u heparinu bila <5%. Srednja korpuskularna krhkost bila je između 0,35 i 0,45% PBS-a u heparinu i 0,85% PBS-a u EDTA. Najjača hemoliza pojavila se u 0,35% PBS-u s EDTA i u 0,20% PBS-u s heparinom. Hematokrit nije bio značajno promijenjen pri pohrani ($P>0,05$). Vrijednosti hematokrita bile su međutim značajno manje u heparinu u usporedbi s EDTA ($P<0,05$). Osmolalnost s EDTA i heparinom bila je 30 minuta nakon uzimanja značajno manja ($P<0,05$) nego nakon šest i 12 sati. Nije bilo značajne razlike u osmolalnosti plazme šest sati nakon uzimanja u usporedbi s vrijednostima nakon 12 sati. Plazma u EDTA imala je značajno veću ($P<0,05$) osmolalnost nego ona u heparinu.

Ključne riječi: noj, antikoagulanti, krhkost eritrocita, hematokrit, osmolalnost