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Inhibition of osmotic permeabilty of caprine erythrocytes by mercuric chloride in osmotic fragility models

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Abstract

Mercuric chloride interferes with erythrocyte membrane and can alter erythrocyte osmotic fragility. Saline and saccharide media have been used in erythrocyte osmotic fragility techniques. Inhibition of erythrocyte osmotic permeability by mercuric chloride was assessed in 10 apparently healthy non-pregnant and non-lactating Sahel does aged two and half years each by dentition. Packed cell volume, erythrocyte count and mean corpuscular volume were determined and calculated using standard methods from heparinised blood from the jugular vein. Erythrocyte osmotic fragility was determined in hypotonic saline, glucose or sucrose medium without and with added mercuric chloride. Erythrocyte parameters were all within normal range for the species. With added mercuric chloride, osmotic stabilization was 84-92% at saline concentrations of 90-300 mOsmol/L, at glucose concentrations of 90-270 mOsmol/L, 9-88% erythrocyte osmotic stabilization was recorded while 39-95% stabilization was observed at sucrose concentrations of 90-270 mOsmol/L. Mercuric chloride inhibited erythrocyte osmotic stability in saline and saccharide media with the highest stabilization in high concentration (300mOsmol/L) of saline, higher stabilization in median (210-270mOsmol/L) and high concentrations of saline than in glucose or sucrose and the least stabilization effect was observed in low (90-180mOsmol/L) and median concentrations of glucose.

Keywords: Glucose, Mercuric chloride, Osmotic stability, Sahel goat, Saline, Sucrose

Introduction

Animals and humans are exposed to heavy metals such as mercury from the environment and food. Mercury is not easily excreted but bioaccumulates and biomagnifies in living tissues (Rice *et al*., 2014). Inorganic mercuric mercury and mercurous mercury are produced from catalase and peroxidase-mediated oxidation of mercury in the blood stream (Huston, 2007). Blood samples containing erythrocytes are easily obtained from living things for diagnostic purposes. Osmotic stability of erythrocytes reflects the ability of the membrane to maintain structural integrity and the membrane redundancy present when the erythrocyte is in equilibrium with an isotonic salt solution (Beutler *et al*., 1982). The rate at which erythrocytes lyse (fragility) is related to their shape, deformability, interaction surface area to volume ratio and intrinsic membrane properties (Bautista *et al*., 2003). Osmotic fragility test is the oldest method of investigating the physical state of erythrocytes. Osmotic fragility test measures erythrocyte resistance to haemolysis when exposed to a series of increasingly dilute saline solutions (Didelon *et al*., 2000; Fernández-Alberti & Fink, 2000). Recently, glucose (Igbokwe & Igbokwe, 2016a) and sucrose (Igbokwe & Igbokwe, 2016b) which are non-ionic media have been used as alternatives to saline a non-ionic medium in the erythrocyte osmotic fragility test which resulted in different interactions with and fluxes of ions across the erythrocyte membrane. The permeability of glucose and non-permeability of sucrose were responsible for different interaction with intracellular components of the erythrocyte.

In humans, i*n vitro* treatment of erythrocytes with low concentrations of mercuric chloride resulted in shrinking of the erythrocytes and conferred protection against osmotic haemolysis (Mel & Reed, 1981), but higher concentration increased erythrocyte osmotic fragility (Lessler & Walters, 1973). Okuda & Tsuzuki (1977) reported decreased erythrocyte osmotic fragility in low doses of methylmercury in male Wistar rats but no change was recorded in erythrocyte osmotic fragility in higher doses. The erythrocytes from female mice fed methylmercury (10nmoles/g feed) had decreased osmotic fragility (Yamamoto & Suzuki, 1982). Erythrocytes treated with mercuric ions showed resistance to osmotic shock after 5 minutes of incubation but they began to haemolyse when the incubation time was increased (Zolla *et al*., 1994).

This study was designed to explore the interaction of mercuric chloride with erythrocyte as to how it affects its stability in ionic (saline) or non-ionic (glucose or sucrose) media and compare stability in either of the non-ionic media and in both ionic and non-ionic media.

Materials and Methods

Ten apparently healthy non-pregnant and non-lactating Sahel does aged two and half years old each were used. They were sourced from housed in the University of Maiduguri animal farm in roofed half-walled pens within a fenced farm area with environmental temperature ranging from 35-38^{o}C. They were offered water and salt lick, fed with cereal offal, grass and legume hays within the pens, and allowed to graze and browse for up to 6 h daily in the surrounding Sahelian bushes outside the fence perimeter.

Blood samples

A blood sample was collected in the morning from each selected animal before leaving the pen. The sample (5 mL) was collected through the external jugular vein using syringe and needle and was put into plastic tubes (Silver Health Diagnostics, Nigeria) containing lithium heparin as anticoagulant. The samples were transported to the laboratory in ice pack and kept within the ice pack without contact with ice until they were analysed within 1-2 hours. Packed cell volume (PCV) and erythrocyte count were determined using microhaematocrit method and haemocytometry, respectively; from which, mean corpuscular volume (MCV) was calculated using a standard formula (Jain, 1993).

Determination of erythrocyte osmotic fragility (EOF)

EOF was determined in a series of hypotonic buffered saline, sucrose or glucose solutions with or without added mercuric chloride. A stock solution of buffered saline was prepared as follows: 90.0 g sodium chloride (NaCl), 13.65 g disodium hydrogen phosphate (Na_2HPO) (BDH, England) and 2.34 g sodium dihydrogen phosphate (NaH_2PO_4) (BDH, England), all made up to 1 L with deionized distilled water, giving a stock solution of 10% NaCl (Ochei & Kolhatkar, 2007). The working solution of 1% NaCl was prepared by dilution of the stock solution from which other lower concentrations (mOsmol/L) of saline were prepared as earlier described (Igbokwe & Igbokwe, 2015). Sucrose and glucose solutions were isosmotic and isotonic at 308 mOsmol/L and prepared as a 105.43 g/L and 55.44 g/L of sucrose and glucose solutions, with the molar masses of sucrose (BDH, Poole, UK) and glucose (BDH, Poole, UK) given as 342.3 g/mol and 180 g/mol respectively. Various dilutions to obtain lower concentrations of sucrose and glucose were made as described by Igbokwe & Igbokwe (2016a) and Igbokwe & Igbokwe (2016b). Mercuric chloride with molecular weight of 271.52g was used to prepare solutions of appropriate mass and osmotic concentrations (0.55-90.00mosmol) in deionized water. The saline, sucrose or glucose solution at various dilutions (5 mL) or saline, sucrose or glucose with various concentrations of mercuric chloride added in a tube had an aliquot of each blood sample (5 µL) added to it, mixed by inversion, and allowed to stand for 30 min under room temperature (35–38 °C). After centrifugation of the tubes at 3000 × g for

15 min, the supernatant of the haemolysate in each tube was harvested with suction pipette into a cuvette, and the haemoglobin colour was estimated as absorbance units with a spectrophotometer (ALL PRO; Shibei, Qingdao, China) set at 540 nm, with the supernatants of the tubes containing corresponding osmolarities devoid of added mercuric chloride and deionised water as blank (0%) and complete (100%) haemolysis, respectively. The calculation of concentrations after dilution process and calculation of degree of haemolysis (%) at each level of dilution were previously described by Igbokwe & Igbokwe (2016a).

Statistical analysis

A coordinate graphing of the dependence of the estimates of haemolysis on concentrations (mOsmol/L) of osmolyte for each blood sample was plotted to obtain the fragility curve. The haemolysis (%) at intervals of 30 mOsmol/L was read on the graph for each blood sample. The concentrations of osmolyte (osmolarities) at 10%–90% haemolysis (CH_{10}–CH_{90}), taken at intervals of 10% haemolysis, on the graph were derived for each blood sample. Data were presented as means ± standard deviations, and means were compared by Student's t-test using computer software (GraphPad InStat, 2013).

Results

The results for erythrocyte parameters are PCV = 31.40±1.20 %, RBC = 12.90±1.86 x10^{12}/L and MCV = 25.00±0.05 fL.

Osmotic stability in saline (ionic) media with added mercuric chloride

The haemolysis of Sahel goat erythrocytes in saline media with and without added mercuric chloride is presented in Figure 1. Haemolysis was significantly ($p < 0.05$) decreased in saline media with added mercuric chloride and osmotic stabilization was 84-92% at saline concentrations of 90-300 mOsmol/L (Table 1). The osmotic concentrations of saline (90-270 mOsmol/L) positively correlated ($r = 0.94$; $p < 0.05$) with the osmotic stabilization elicited by added mercuric ion.

Osmotic stability in glucose or sucrose (non-ionic) media with added mercuric chloride

The haemolysis of Sahel goat erythrocytes in glucose media with and without added mercuric chloride is presented in Figure 2. Haemolysis was significantly ($p < 0.05$) decreased in glucose media with added mercuric chloride. Osmotic destabilization (68%) was observed when mercuric ions were added to glucose media at 300 mOsmol/L (Table 2). Osmotic stabilization indices were 9-88% at glucose concentrations of 90-270 mOsmol/L (Table 2). Low osmotic stabilization (≈9%) by mercuric ions occurred at high glucose concentrations of 240-270 mOsmol/L. A moderate rise in osmotic stabilization to 50% was observed as the glucose concentration dropped to 210 mOsmol/L and at the point where the glucose concentration was ≤180 mOsmol/L, the osmotic stabilization was >86%. The osmotic concentrations of glucose (90-270 mOsmol/L) negatively correlated ($r = -0.90$; $p < 0.05$) with the

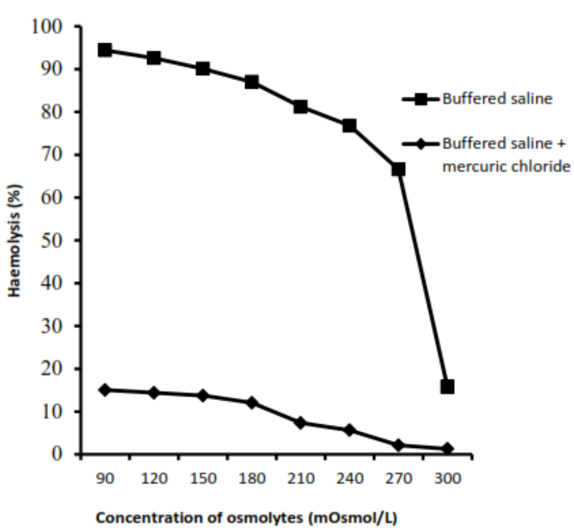

Figure 1: Fragility curves showing haemolysis of Sahel goat erythrocytes in saline alone and its combination with mercuric chloride

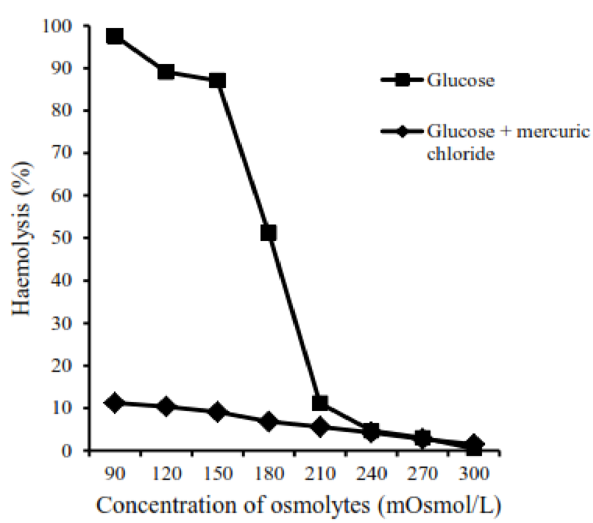

Figure 2: Fragility curves showing haemolysis of Sahel goat erythrocytes in glucose alone and its combination with mercuric chloride

Table 1: Haemolysis in various concentrations of saline or its combination with mercuric chloride (0.46 mOsmol/L)

Concentration of osmolytes (mOsmol/L)	Haemolysis (%) in		Osmotic stabilization (%)
	Saline	Saline + $HgCl_2$	
300	15.80±2.95[a]	1.30±0.32[b]	91.77
270	66.60±12.64[a]	2.12±0.34[b]	96.82
240	76.80±12.11[a]	5.66±3.86[b]	92.63
210	81.20±12.59[a]	7.34±5.16[b]	90.96
180	87.00±6.75[a]	12.07±3.34[b]	86.13
150	90.10±6.71[a]	13.74±2.33[b]	84.75
120	92.58±5.38[a]	14.40±1.93[b]	84.85
90	94.40±4.72[a]	15.06±1.49[b]	84.05

[a,b]Means ± standard deviations with different superscripts are significantly ($p<0.05$) different

Table 2: Haemolysis in various concentrations of glucose or its combination with mercuric chloride (0.46 mOsmol/L)

Concentration of osmolytes (mOsmol/L)	Haemolysis (%) in		Osmotic stabilization index (%)	Osmotic destabilization index (%)
	Glucose	Glucose + $HgCl_2$		
300	0.48±0.39[a]	1.48±0.34[b]	0	67.57
270	2.98±1.19[a]	2.72±0.47[a]	8.72	0
240	4.68±1.80[a]	4.26±0.57[a]	8.97	0
210	11.16±8.04[a]	5.60±0.76[a]	49.82	0
180	51.18±5.85[a]	6.84±1.00[b]	86.64	0
150	87.06±3.25[a]	9.10±0.51[b]	89.55	0
120	89.06±3.25[a]	10.36±0.90[b]	88.37	0
90	97.54±1.29[a]	11.24±0.95[b]	88.48	0

[a,b]Means ± standard deviations with different superscripts within the rows are significantly ($p<0.05$) different

osmotic stabilization elicited by added mercuric ion. negatively correlated ($r = -0.90$; $p < 0.05$) with the osmotic stabilization elicited by added mercuric ion.

The haemolysis of Sahel goat erythrocytes in sucrose media with and without added mercuric chloride is presented in Figure 3. Estimates of haemolysis were significantly ($p < 0.05$) decreased in sucrose media with added mercuric chloride and osmotic stabilization was 39-95% at sucrose concentrations of 90-270 mOsmol/L (Table 3). However, osmotic destabilization (76%) was observed at 300 mOsmol/L of sucrose media. The osmotic concentrations of sucrose (90-270 mOsmol/L) negatively correlated ($r = -0.90$; $p < 0.05$) with the osmotic stabilization elicited by added mercuric ion.

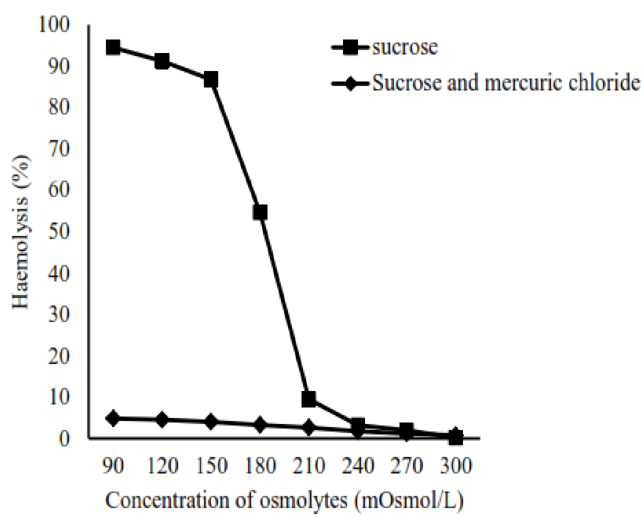

Figure 3: Fragility curves showing haemolysis of Sahel goat erythrocytes after incubating in sucrose alone and its combination with mercuric chloride

Comparison of the osmotic stabilization of erythrocytes by mercuric chloride added to ionic or non-ionic media

The comparison of osmotic stabilization indices of erythrocytes of Sahel goats by mercuric chloride added to ionic or non-ionic media are presented in Table 4. The aggregate data show that mercerized saline media produced a significantly ($p < 0.05$) higher osmotic stabilization than mercurized glucose or sucrose media at medium (210-270 mOsmol/L) concentrations of media. A significantly ($p < 0.05$)

Table 3: Haemolysis in various concentrations of sucrose or its combination with mercuric chloride (0.46 mOsmol)

Concentration of osmolytes (mOsmol/L)	Haemolysis (%) in		Osmotic stabilization (%)	Osmotic destabilization (%)
	Sucrose	Sucrose + $HgCl_2$		
300	0.18±0.20[a]	0.74±0.33[b]	0	75.6
270	1.96±0.67[a]	1.20±0.36[a]	38.78	0
240	3.20±0.91[a]	1.76±0.48[b]	45.00	0
210	9.46±2.57[a]	2.63±1.10[b]	72.20	0
180	54.60±3.13[a]	3.28±1.57[b]	93.99	0
150	86.80±1.75[a]	4.03±1.46[b]	95.37	0
120	91.22±2.98[a]	4.54±1.31[b]	95.02	0
90	94.50±2.24[a]	4.85±1.15[b]	94.87	0

[a,b]Means ± standard deviations with different superscripts within the rows are significantly ($p<0.05$) different

Table 4: Comparison of the osmotic stabilization of erythrocyte by mercuric chloride added to ionic or non-ionic media

Osmolytes	Osmotic stabilization (%) at various concentrations of media (mOsmol/L)		
	300 (High)	210-270 (Median)	90-180 (Low)
Saline + $HgCl_2$	91.77	93.47±3.02[a]	84.95±0.80[a]
Glucose + $HgCl_2$	0	22.50±23.65[b]	88.26±1.20[b]
Sucrose + $HgCl_2$	0	51.99±17.97[c]	94.82±0.59[c]

[a,b,c]Means±standard deviations with different superscripts are significantly ($p<0.05$) different down the column

lower stabilization was observed in mercurized saline than glucose or sucrose media at low (90-180 mOsmol/L) concentrations of media. At high media concentration (300 mOsmol/L) mercurized saline stabilized erythrocytes by 92%, but no stabilization occurred in mercurized glucose or sucrose media where there was rather a destabilization of 68% or 76%, respectively. There was a greater stabilization by mercuric ions in sucrose than in glucose media from 90-270 mOsmol/L. The relationship between osmotic stabilization of erythrocytes in mercurized saline and the media concentrations in which it occurred was a direct correlation, but this relationship was changed to an inverse correlation in mercurized glucose or sucrose media.

Discussion

The values for PCV, RBC and MCV were all within the normal range for the species (Jain, 1993) indicating that the does were healthy and erythrocyte osmotic fragility was not affected by variations of these parameters. Erythrocyte osmotic stability increased in various concentrations of media containing saline, glucose or sucrose upon the addition of mercuric chloride to each medium except at high concentrations of glucose and sucrose where it caused a destabilization. The destabilization observed could be due to molecular crowding, increased osmotic pressure and mercuric-induced oxidative stress to erythrocyte membrane (Ribarov *et al*., 1983; Lund *et al*., 1993; Sharma *et al*., 2007; Augusti *et al*., 2008; Durak *et al*., 2010) as a result of permeability of mercury across the membrane (Bienvenue *et al*., 1984). Mercury does reduce the action of antioxidant enzymes (Bansal *et al*., 1992; Park & Park, 2007) and cause lipid peroxidation (Bansal *et al*., 1992; Lund *et al*., 1993; Mahboob *et al*., 2001; Augusti *et al*., 2008; Durak *et al*., 2010). The protection conferred on erythrocyte membrane against oxidative damage by enzyme activities involved in the antioxidant systems was disrupted by mercury (Queiroz *et al*., 1998) and made the membrane susceptible to damage. Glutathione which is the most abundant intracellular antioxidant in the erythrocyte and is involved in glutathione peroxidase reaction needed for protection of haemoglobin against oxidation (Hayes & McLellan, 1999; Waggiallah & Alzohairy, 2011) has been reported to be reduced by mercury in human erythrocytes (Weed *et al*., 1962).

The increased erythrocyte osmotic stability observed in this study could be attributed to mercury-induced oxidative stress reported to increase levels of intracellular Ca^{2+} through Ca^{2+} permeable cation channels leading to elevated levels of Ca^{2+}-sensitive scramblase and spingomyelase (Shetthalli & Gummadi, 2013). Increased scramblase activity changed lipid asymmetry and exposed phosphatidylserine in erythrocyte membrane (Eisele *et al*., 2006; Kyung-Min *et al*., 2010; Shettihalli &

Gummadi, 2013). Oxidative stress arising from metallic toxicant such as mercury could induce eryptosis (Lang *et al*., 2014; Lang & Lang, 2015a, 2015b). *In vitro* treatment with mercuric chloride could have made the erythrocytes to be eryptotic, shrunken, resisted haemolysis and improved osmotic stability. Similarly, exposure of human erythrocytes to mercury induced an efflux of intracellular K^+ with obliged water movement with most of the cells presenting as echinocytes (Suwalsky *et al*., 2000; Brandon *et al*., 2015). Mercuric ions bind with sulfhydryl groups in cell membranes to alter both active and passive transport of Na^+ and K^+ (Zolla *et al.,* 1997) and enzymes by distorting their shape and activities (Zalups, 2000).

Interactions between mercury and phospholipids like phosphatidylcholine, phosphatidyl serine and phosphatidyl etahnolamine in the erythrocyte membrane (Girautt *et al*., 1996; Suwalsky *et al*., 2000; Delnomdedieu & Allis, 1993; Delnomdedieu *et al*., 1989; Delnomdedieu *et al*., 1992) form a gel that protects the membrane. Mercuric ions scramble phospholipids in erythrocyte membrane (Van Zwieten *et al*., 2012) and improve the activities of factors involved in coagulation (Kyung-Min *et al*., 2010; Shetthalli & Gummadi, 2013) and prevent haemolysis by reducing influx of mercuric ions into the erythrocytes. Influx of mercuric ions into the erythrocytes would increase susceptibility to oxidative stress. Low levels of oxidative injury caused eryptosis, whereas more severe oxidative stress caused oncotic necrosis with decreased osmotic stability. Low level of osmotic destabilization with mercuric ions at high sucrose media concentration indicated that mercuric ions induced haemolysis enhanced by sucrose associated molecular crowding. Treatment with mercury reduced aceytlcholinesterase activity in Wistar rat (Miszta, 1984) and human erythrocytes (Kyung-Min *et al*., 2010). The type of acetylcholinesterase in erythrocyte membrane and how it binds to mercury is species specific (Frasco *et al*., 2007) even though its role in the membrane is unclear (Lawson & Barr, 1987). Mercuric ions might have also interfered with water channels by either changing the conformation of proteins or by steric mechanism (Savage & Stroud, 2007) or reduced water permeability across erythrocyte membrane (Yakutake *et al*., 2008). Aquaporins are mercury-sensitive water channels and play a significant role in swelling when erythrocytes are suspended in hypotonic media (Pribush *et al.,* 2002). In the presence of mercuric ions, aquaporin function might have been reduced.

In conclusion, mercuric chloride improved erythrocyte osmotic stability in low and median concentration of saline and saccharide media. Stabilization was highest in high concentration of saline, higher in median and high concentration of saline than in glucose or sucrose and the least stabilization effect was observed in low and median concentration of glucose. Stabilization effect of mercuric chloride was higher in sucrose than in glucose in both low and median concentration of media but there was no stabilization effect in high concentration of glucose or sucrose.

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