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Erythrocyte Membrane Stability after Transportation Stress in the Domestic Chicken as Modulated by Pretreatment with Vitamins C and E

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Abstract: The effects of transportation stress and pretreatment with non-enzymic antioxidants vitamins C and E on the erythrocyte osmotic fragility, haematocrit, haemoglobin, red blood cell and white blood cell counts, Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC) and the differential leucocyte counts were investigated using the domestic chicken. About 32, adult female domestic chickens of the Nera black strain were used. The birds were divided into four groups A-D consisting of 8 birds each. Groups A and B did not receive any medication. Group C received vitamin C (650 mg kg^{-1} diet) while D, vitamin E (270 mg kg^{-1} diet) in feed for 2 weeks. Birds in groups B, C and D were transported through a distance of 200 km. Blood samples were collected within 30 min after the journey for analysis. Contrary to the expectation, the erythrocyte osmotic fragility was lower in the transported, untreated birds than the control while those pretreated with vitamins C and E had higher osmotic fragility. Haemoglobin and MCHC values were higher in the transported, untreated while other parameters were similar. This study demonstrated that nucleated erythrocytes of the domestic chicken unlike other animals with non nucleated erythrocytes, respond to acute stress by increase in osmotic resistance and membrane stability in hypotonic solution, this was antagonized by pretreatment with vitamins C and E.

Key words: Transportation, pretreatment, domestic chicken, stability, expectation, Nigeria

INTRODUCTION

Exposure to various forms of stress such as exercise stress, heat and cold stress, ionizing radiation, transportation stress, prolonged restraint etc have been shown to be deleterious to health and well being of both man and animals. This is unconnected with increased generation of free radical and other reactive oxygen species in these conditions (Kelle *et al*., 1999; Ozturk and Gumuslu, 2004; Sahin *et al*., 2004; Powers and Jackson, 2008). But these stressor factors are part of mans day to day activities, some of which are very difficult or practically impossible to avoid totally. This is especially true of stress associated with transportation. Transportation being a daily activity in man and domestic animals has been shown to be stressful to animals this is usually seen as increased excitability, increased heart and respiratory rates, dehydration (coupled with increased plasma osmolality, increased PCV), physical exertion, elevated cortisol and vasopressin levels in the blood leading to motion sickness and elevated body temperature (Broom, 2005).

Various forms of stress factor such as exercise, heat and transportation stress as well as aging which lead to lipid free radicals and lipid peroxides generation in membranes make a major contribution to ROS-induced injury. An initiator (such as a hydroxyl radical produced locally in the Fenton reaction) begins the chain reaction. It extracts a hydrogen atom, preferably from the double bond of a polyunsaturated fatty acid in a membrane lipid. The chain reaction is propagated when O_2 adds to form lipid peroxyl radicals and lipid peroxides. Eventually lipid degradation occurs, forming such products as malondialdehyde (from fatty acids with three or more double bonds) and ethane and pentane (from the ω-terminal carbons of 3 and 6 fatty acids, respectively). Malondialdehyde appears in the blood and urine and can be used as an indicator of free radical damage. Peroxidation of lipid molecules invariably changes or damages lipid molecular structure. In addition to the self-destructive nature of membrane lipid peroxidation, the aldehydes that are formed can cross-link proteins. When the damaged lipids are the constituents of biologic membranes, the cohesive lipid bilayer arrangement and stable structural organization is disrupted. This disruption may lead to increased permeability to water and Ca^{2+} and osmotic swelling of erythrocytes in hypotonic solution.

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Cells protect themselves against damage by ROS and other radicals through repair processes, compartmentalization of free radical production, defense enzymes and endogenous and exogenous antioxidants (free radical scavengers). The defense enzymes, Superoxide Dismutase (SOD) removes the superoxide free radical. Catalase and glutathione peroxidase remove hydrogen peroxide and lipid peroxides. Vitamin E, vitamin C and plant flavonoids act as antioxidants.

Although, Vitamin C is an oxidation-reduction coenzyme that functions in collagen synthesis and other reactions, it also plays a role in free radical defense. Reduced ascorbate can regenerate the reduced form of vitamin E through donating electrons in a redox cycle. It is water-soluble and circulates unbound in blood and extracellular fluid, vitamin E (α-tocopherol), the most widely distributed antioxidant in nature is a lipid-soluble antioxidant vitamin that functions principally to protect against lipid peroxidation in membranes. Vitamin E comprises a number of tocopherols that differ in their methylation pattern. Among these, α-tocopherol is the most potent antioxidant and present in the highest amount in the diet. Vitamin E is an efficient antioxidant and non-enzymatic terminator of free radical chain reactions and has little pro-oxidant activity.

In this study, the effect of transportation stress was evaluated on the erythrocytes of the domestic chicken by measurement of the cell stability in hypotonic solution. Since exposure of erythrocytes to oxidative stress and free radicals can induce hemoglobin damage and stimulate protein degradation, lipid peroxidation and haemolysis (Girotti, 1985). Erythrocyte osmotic fragility therefore is a good indicator of the level of oxidative damage from transportation stress (Adenkola and Ayo, 2009). According to Kraus *et al.* (1997), vitamins C and E supplementation reduced erythrocyte osmotic fragility and oxidative damage in rats, the effects of supplementation of these non enzymic antioxidants was then evaluated on the erythrocyte osmotic fragility of domestic chicken of the Nera black strain.

MATERIALS AND METHODS

About 32 adult 20 week old, female Nera black chickens obtained from a commercial farm in Ibadan, Nigeria were divided into four groups A, B, C and D consisting of 8 birds per group. They were allowed to stabilize for 2 weeks during which they were dewormed with piperazine dihydrate (Alfasan, Holland) at 0.1 g L^{-1} of drinking water followed by prophylactic treatment with an antibiotic, NCO Mix® (Kepro B.V, Holland) containing neomycin, chloramphenicol and oxytetracycline which was administered at a dose rate of 0.25 g L^{-1} of drinking water for 5 days. Grower mash feed (Vita Feed Ltd, Ibadan, Nigeria) and water was provided *ad libitum* throughout the period. The birds in groups C and D were given Vitamins C (650 mg kg^{-1} of feed) and E (α-tocopherol at 270 mg kg^{-1} of feed), respectively according to Kraus *et al.* (1997) for 2 weeks while those in groups A and B did not receive any medication. After 2 weeks, the birds in groups B, C and D were put in metal cages according to their groups for adequate comfort and ventilation. They were transported early in the morning to and fro Lagos from the Experimental Animal Unit of the Faculty of Veterinary Medicine, University of Ibadan, Ibadan, covering a distance of 200 km on the highway. The birds in group A which served as the control were not transported.

Blood sample (5 mL each) was collected through the jugular veins into heparinized tubes from all the 32 birds, 30 min after returning from the journey and analyzed immediately.

From the blood samples, the Packed Cell Volume (PCV) was determined by microhaematocrit method. Red Blood Cells (RBC) and White Blood Cells (WBC) were counted using the haemocytometer method. Haemoglobin concentration (Hb) was determined by the cyanmethaemoglobin method. The Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH) and Mean Corpuscular Haemoglobin Concentration (MCHC) were calculated from the PCV, RBC and Hb values.

Erythrocyte osmotic fragility was determined by adding 0.02 mL of blood to tubes containing increasing concentration of phosphate-buffered sodium chloride (NaCl) solution at pH 7.4 (0, 0.1, 0.3, 0.5, 0.7, 0.8 and 0.9%) as described by Oyewale. The tubes were gently mixed and incubated at room temperature (29°C) for 30 min. The content of each tube was then centrifuged at 3500 rev min^{-1} for 10 min and the supernatant decanted. Optical density of the supernatant was determined spectrophotometrically at 540 nm using SM22PC Spectrophotometer (Surgienfield Instruments, England). Haemolysis in each tube was expressed as a percentage, taking haemolysis in distilled water (0% NaCl) as 100%.

Statistical analysis: All data were compared by One way ANOVA, using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). The value of $p<0.05$ was considered significant.

RESULTS AND DISCUSSION

The effects of transportation stress on haematological parameters of the domestic chicken pretreated with vitamins C and E is shown in Table 1. There was no significant change in the Packed Cell Volume (PCV), Red Blood Cell (RBC) and White Blood Cell (WBC) Counts, Mean Corpuscular Volume (MCV) and Mean Corpuscular Haemoglobin (MCH). The differential leucocyte counts also did not exhibit any significant change in all the groups of birds transported over a distance of 200 km. The Haemoglobin concentration (Hb) and the Mean Corpuscular Haemoglobin Concentration (MCHC) however were significantly higher ($p<0.05$) in the birds in group B (those transported but not treated with any antioxidants) than those of the control (Group A) that were not exposed to transportation stress.

The erythrocyte osmotic fragility of the birds exposed to transportation stress without any antioxidant (Group B) was lower (Fig. 1) than those of the control at 0.1 and 0.3% NaCl concentration. In actual fact, 75 and 50% haemolysis occurred at NaCl concentrations of 0.15 and 0.33%, respectively in the control while 75 and 50% haemolysis occurred at NaCl concentrations of 0.08 and 0.22% in the birds in group B (Fig. 1). In the groups pretreated with the antioxidants, the erythrocytes osmotic fragility of the birds treated with vitamin C (Group C) was initially lower ($p<0.05$) than were those of either the control or those in group B at 0.0% NaCl concentration (Fig. 2 and 3) but the fragility significantly increased at 0.1, 0.3 and 0.9% NaCl concentrations. It was higher than those of group B at 0.1% NaCl, those of groups A and B ($p<0.05$ and $p<0.01$, respectively) at 0.3% NaCl. It was also higher ($p<0.05$) than either of group A or B at 0.9% NaCl. The osmotic fragility of the birds pretreated with vitamin E was also higher ($p<0.05$) than that of the untreated birds that were exposed to transportation stress (group B) at 0.3% NaCl concentration (Fig. 2 and 3).

Table 1: Haematological parameters of the domestic chicken pretreated with vitamins C and E after transportation stress. Values are means±SEM. Number of birds in parenthesis

Parameters	Group A (8) (Not transported)	Group B (7) (Transported)	Group C (8) (Transported+ vitamin C)	Group D (8) (Transported+ vitamin E)
PCV (%)	31.71±1.980	31.40±0.740	29.83±0.850	33.57±1.190
RBC ($\times10^6$ μL^{-1})	3.20±0.240	3.43±0.090	3.07±0.220	3.67±0.140
Hb (g dL^{-1})	10.43±0.690	12.28±0.280*	9.98±0.250	11.00±0.310
MCV fl	101.0±5.9500	91.54±2.080	97.30±4.010	91.61±0.790
MCH (pg)	33.16±1.920	29.97±0.800	33.26±1.650	30.07±0.420
MCHC (g dL^{-1})	30.89±1.100	39.11±2.650*	33.46±4.010	32.76±0.790
Platelets ($\times10^3$ μL)	168.00±25.33	181.20±26.21	189.17±30.44	227.29±31.43
WBC ($\times10^3$ μL)	15.98±0.630	11.35±0.470	16.28±1.220	15.75±0.740
Eosinophils ($\times10^3$ μL)	0.60±0.070	0.44±0.030	0.53±0.060	0.59±0.100
Monocytes ($\times10^3$ μL)	0.32±0.060	0.31±0.080	0.48±0.080	0.39±0.060
Heterophils ($\times10^3$ μL)	4.49±0.450	3.34±0.430	4.15±0.640	4.58±0.650
Lympho ($\times10^3$ μL)	10.57±0.640	7.26±1.070	11.12±1.310	10.18±1.140
H/L ratio	0.42±0.250	0.46±0.150	0.41±0.170	0.45±0.200

Asterisk indicate significant difference from the control, *$p<0.05$

The erythrocytes of the domestic chicken (Nera black strain) in this study responded to transportation stress by decrease in osmotic fragility as shown in Fig. 1. This is

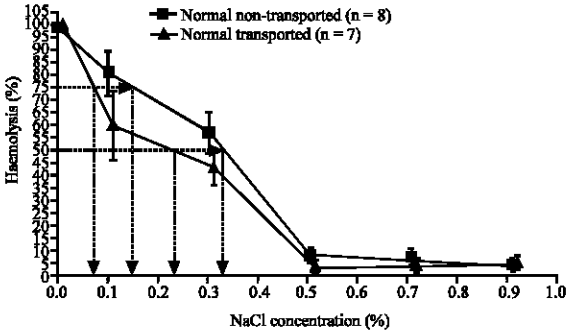

Fig. 1: Effects of transportation stress on the erythrocyte osmotic fragility of the domestic chicken. Values are means and vertical bars represents SEM. Number of birds in parenthesis

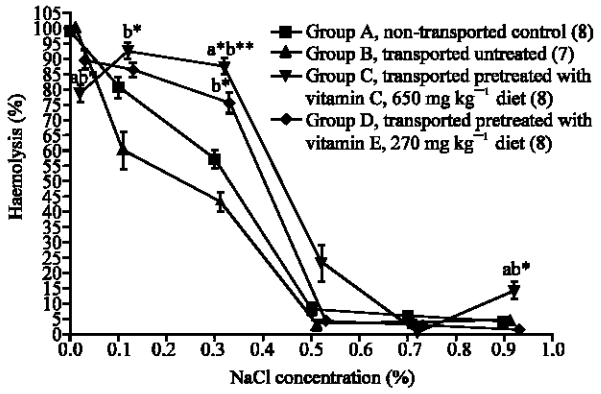

Fig. 2: Erythrocyte osmotic fragility of the domestic chicken (*Gallus domesticus*) showing the effects of transportation stress and pretreatment with vitamins C and Normal transported E. Values are means and vertical bars represent SEM. Number of birds in parenthesis; [a]indicate significant difference from the untransported birds (Group A), [b]indicate significant difference from the transported, untreated birds (Group B), * $p<0.05$, ** $p<0.01$

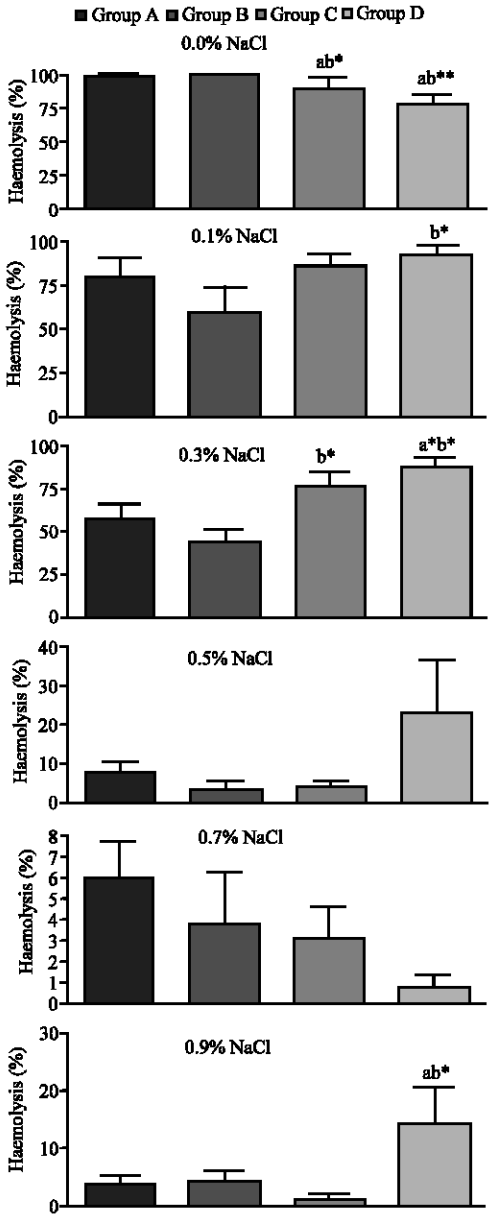

Fig. 3: Erythrocyte osmotic fragility of the domestic chicken (*Gallus domesticus*) pretreated with vitamins C and E, after transportation stress at different NaCl concentration, [a]indicate significant difference from the transported, untreated birds (Group B), *p<0.05,** p<0.01

contrary to previous reports on the effects of oxidative stress on the erythrocyte osmotic fragility in other animal species. For instance, Adenkola and Ayo (2009) reported increased erythrocyte osmotic fragility in pig as result of transportation stress although, the fragility was reduced by pretreatment with vitamin C. Similarly, exercise stress, heat stress and other oxidative stress have been associated with increased erythrocyte osmotic fragility, concurrently with elevated levels of Thiobarbituric Acid Reacting Substances (TBARS) and Malondialdehyde (MDA) which are products of lipid peroxidation in the erythrocyte membrane. Also associated with oxidative stress are reductions in the levels of intra-erythrocytic Cu, Zn and Mn-Superoxide Dismutase (SOD), glutathione peroxidase and elevated catalase and oxidized glutathione levels (Kelle *et al*., 1999; Ozturk and Gumuslu, 2004; Aguilo *et al*., 2005). Damage to the erythrocyte membrane proteins and lipids then leads haemolysis as a result of destruction of the spectrin bands which is the base of the erythrocyte cytoskeleton. Also damaged by the oxidative stress according to Reid and Mohandas (2004) are band 3, glycophorin C and RhAG, the membrane proteins that link the lipid bilayer to the spectrin cytoskeleton. These linkages play significant role in regulating cohesion between the lipid bilayer and the cytoskeleton; loss of which results in lipid loss, decreased membrane surface area and loss of deformability of erythrocytes.

It thus appears that nucleated erythrocytes of the Nera black chicken respond to oxidative stress by decreased fragility in hypotonic solution. This is contrary to increased fragility previously observed in the same strain of domestic chicken as a result of aging (Azeez *et al*., 2009), it is however, similar to the previous observation in rainbow lizard (*Agama agama*) that exhibited decreased erythrocyte fragility after swimming exercise (Azeez and Oyewale, 2010). The observation in the present study may be due to the shortness of the distance covered or an adaptation to oxidative damage by Nera black chicken. Smith *et al*. (1989) and Hanzawa *et al*. (1996) had previously reported significant decreases in erythrocyte fragility during short duration or low intensity exercises in thoroughbred horses. It was postulated that the reduced erythrocyte osmotic fragility in these horses was due to shrinkage of erythrocytes as a result of increased activities of K-Cl co-transporters in the erythrocyte membrane to prevent intravascular haemolysis during exercise (Hanzawa *et al*., 2004).

It must however be noted that reduction in membrane fragility due to shrinkages will inadvertently lead to membrane rigidity and loss of deformability of erythrocytes (Knowles *et al*., 1994). This predisposes the erythrocytes to sequestration and destruction in the spleen (An and Mohandas, 2008). This may partly explain the higher haemoglobin concentration in the birds in group B. Pretreatment with vitamin C and E obviously antagonized the effect of the transportation stress in this study. Ascorbic acid and α-tocopherol have been known from ages as non enzymatic antioxidants, restoring

erythrocyte osmotic resistance after stress (Kelle *et al*., 1999; Adenkola and Ayo, 2009). Ascorbic acid (vitamin C) acts by donating hydrogen atom to lipid radicals, quenching singlet oxygen and removal of molecular oxygen. It also participates in the regeneration of α-tocopherol. Vitamin E on the other hand is a natural constituent of biological membranes; it acts as an antioxidant by donating hydrogen atom at 6-hydroxyl group on the chromatin ring and by scavenging singlet oxygen and other reactive species (Lee *et al*., 2004; Powers and Jackson, 2008).

CONCLUSION

This study has shown that Nera black chicken respond to transportation stress by decreased erythrocyte osmotic fragility leading to increased resistance to osmotic lysis. Transportation stress also increased the haemoglobin concentration and the mean corpuscular haemoglobin concentration. The osmotic fragility was however modulated by pretreatment with non enzymic antioxidant vitamins C and E.

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