Full Length Research Paper

Changes in Hematological Parameters and Erythrocyte Osmotic Fragility in Lame and Aged Horses Administered with Resveratrol Supplement

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ABSTRACT

The study was aimed at evaluating the changes in haematological parameters and erythrocyte osmotic fragility in lame and aged horses administered with resveratrol supplement (Equithrive joint[®]). A total of 16 horses of both sexes, aged 18 ± 0.65 and showing lameness grade 3 were used for the study. The horses weighed 350-450 kg and comprised 8 horses which were administered with resveratrol supplement for 4 weeks and 8 others, which served as controls and given only *Saccharomyces cerevisiae* yeast strain used as carrier in the supplement. Blood samples were collected from each horse before supplementation and at weekly intervals for 4 weeks of the experiment. Haematological parameters and erythrocyte osmotic fragility were determined by standard methods. Equithrive joint[®] increased significantly ($P < 0.05$) packed cell volume, haemoglobin concentration and erythrocyte counts in the treated horses while total leucocyte, neutrophil and eosinophil counts decreased significantly ($P < 0.05$) in the treated horses compared with the untreated horses. Erythrocyte osmotic fragility test showed decreased haemolysis in the treated horses. The result indicated that equithrive joint[®] a potent antioxidant and anti-inflammatory agent maintained the membrane integrity of red blood cells and may be of value in aiding horses move with ease during ageing.

Keywords: Horses, erythrocyte osmotic fragility, haematological parameters, resveratrol, ageing, lameness

INTRODUCTION

Haematological parameters are good indicators of the physiological status of animals (Lassen and Swardson, 1995), widely reported to vary as animals advance in age (Satué *et al*., 2009). Reduction in erythrocytes has been associated with ageing (Hernández *et al*., 2008) due to reduced regenerative capacity of the bone marrow (McFarlane *et al.,* 1998). Lymphocyte counts decrease during adulthood, while neutrophil counts remains the same, resulting in a higher neutrophil/lymphocyte ratio in aged horses compared to foals (Jain, 1986). It is an established fact that an increase in the ratio is a good indicator of stress (Minka and Ayo 2007).

Degenerative bone and joint diseases are associated with oxidative stress particularly as animals advanced in age (Hekimi *et al.,* 2011). This is due to generation of

reactive oxygen species (ROS) (Speakman and Selman, 2011). ROS produced during stress is known to play essential role in tissue damages and exert adverse effects on RBCs (Gümülü *et al.,* 2002).

Erythrocyte osmotic fragility (EOF) is an important index of quantifying oxidative stress indirectly in livestock (Adenkola *et al.,* 2010).

In order to neutralize the threat of ROS, a wide variety of antioxidants has been evolved (Halliwell, 2012). They include the natural dietary compounds found in a variety of fruits, vegetables, nuts and seeds, which combat the adverse effects of oxidative stress.

Equithrive joint®, a resveratrol preparation for horses, contains a high-quality research-proven source of resveratrol, which is easily administered orally (Lawless, 2010). Resveratrol (3,5,4'-trihydroxystilbene), is a polyphenol found in grapes (Vitis vinifera), a variety of berries, peanuts and medicinal plants, such as Japanese knotweed (Polygonum cuspidatum) (Pervaiz, 2003). It has received much attention for its anti-inflammatory and antioxidant properties, and its ability to increase lifespan in lower organisms and improve general health in mammals (Baur and Sinclair, 2006). Equithrive joint®, also contains sodium hyaluronic acid, the primary component of joint, which lubricates synovial fluid and cartilage (Michele and Vincenzo, 2012).

The aim of the study was to determine the changes in haematological parameters and EOF in lame and aged horses administered with resveratrol supplement.

MATERIALS AND METHODS

Experimental site and design
The study was carried out in a polo farm in Kaduna (10º 29′ N, 07º 28′ E), located in the Northern Guinea Savannah zone of Nigeria. It involved 16 horses of both sexes, aged between 15 and 22 years, weighing between 350-450 kg and showing lameness grade 3; that is, lameness consistently observed at a trot in all circumstances (Stashak, 1987). They were randomly assigned to treated and untreated (control) groups of 8 animals each. The horses were housed in standard stables measuring 10 m × 12 m, made of concrete floor, cement block wall and asbestos roof, and well ventilated. The horses were fed with wheat bran, sorghum, hay and fresh pasture. They were pre-conditioned for 2 weeks before the commencement of the supplementation; and during this period, they were screened and treated for endoparasites and hemoparasites

Resveratrol supplement (Equithrive joint®) was purchased from Hagyard Pharmacy, Kentucky, USA. Treated horses were fed 30 g of equithrive joint powder as loading dose for the first ten days of the experiment, followed by 15 g of equithrive joint® powder as maintenance dose for the remaining 18 days of the study. The untreated horses were fed 30 g of *Saccharomyces cerevisiae,* which is the carrier of the equithrive joint powder for the first ten days of the experiment, and then 15 g of *Saccharomyces cerevisiae* for the remaining 18 days of the study. The supplement was mixed in their daily feed during the period of the study (Horokov and Adams, 2008). Both the treatment and control groups received equal amount of their normal feed each day of the study period. All horses were fed twice daily and monitored during feed consumption and also maintained on the same pasture, and given access to water *ad libitum.*

Blood Sample Collection
This was carried out during the two weeks pre-conditioning period to obtain base-line data and then at weekly intervals for 4 weeks of treatment. Blood sample (6 ml) was collected from each animal in the morning before feeding by jugular venipuncture using disposable syringes and 18 gauge x 1.5 inch sterile needles. Each blood sample collected was divided in two parts; 3 ml of blood was dispensed in tube containing ethylenediaminetetra acetic acid (EDTA) for determination of red blood cell count (RBC), haemoglobin (Hb) concentration, packed cell volume (PCV) and total and differential white blood cell (WBC) counts. The remaining 3 ml was placed in tube, containing sodium citrate in order to evaluate the EOF.

Blood sample analysis
RBC, Hb, PCV, total and differential WBC counts were determined as described by Dawies and Lewis (1991). The EOF was determined using the method described by Oyewale et al. (2011). Briefly, Sodium chloride (NaCl) stock solution (pH 7.4) was prepared in volumes of 500 ml for each of the samples in concentration of 0.1%, 0.3%, 0.5%, 0.7%, 0.9%. Each of the five test tubes contained 5 ml of the corresponding NaCl concentration from the stock solution. The test tubes were labeled with corresponding concentrations and arranged serially in a rack of five tubes. Pipette (1 ml) was used to transfer exactly 0.02 ml of each blood sample into each of the five test tubes in a set. The contents of the test tubes were gently mixed by inverting the test tubes five times and allowing them to stand at room temperature (25ºC) for 30 minutes. Thereafter, the contents of the test tubes were centrifuged at 1500 x g for 15 minutes. The

supernatant was transferred into glass cuvette and measured at wavelength of 540 nm using a spectrophotometer (Spectronic-20, Philip Harris Limited®, Shenstone, UK) by reading the absorbance. The percentage haemolysis in each concentration of NaCl was determined by taking the tube with maximum haemolysis (0%) as 100%.

Data Analysis

A graph pad prism version 4.0 windows (GraphPad Software, San Diego, California, USA) was used. Data obtained were expressed as mean ± standard error of mean (Mean ± SEM) and were subjected to student's *t*-test to determine the difference between treated and untreated horses at each period of sampling. Repeated measures ANOVA and Tukey's *post-hoc* test were used to determine the effects of sampling periods. Values of $P < 0.05$ were considered significant

RESULTS

The RBC counts were significantly ($P < 0.05$) higher in treated than untreated horses on the third week of administration of resveratrol supplement (Table 1). The haemoglobin concentration was also higher ($P < 0.05$) in treated horses than untreated group horses on second and third weeks of supplementation. The PCV values rose in the treated horses from $35.4 \pm 2.42\%$ to $43.1 \pm 2.3\%$ and $45.6 \pm 2.3\%$ on second and third week respectively. The values were higher ($P < 0.05$) than the corresponding values of $35.0 \pm 2.46\%$ to $36.0 \pm 2.3\%$ and $39.8 \pm 1.1\%$ recorded in the control horses.
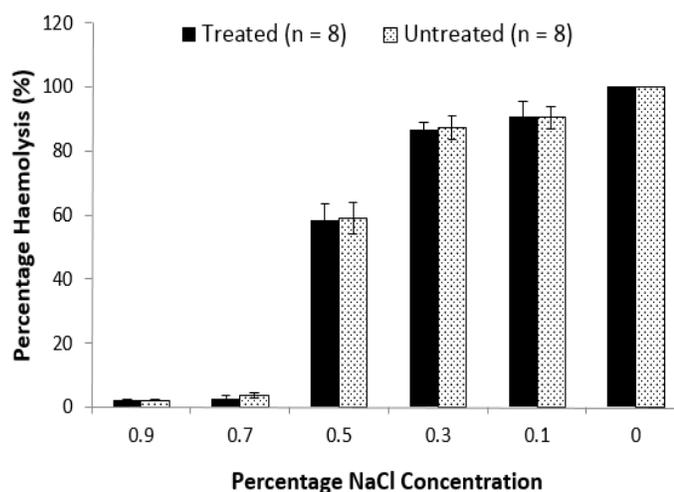
Fig. 1:
Erythrocyte osmotic fragility of horses before supplementation
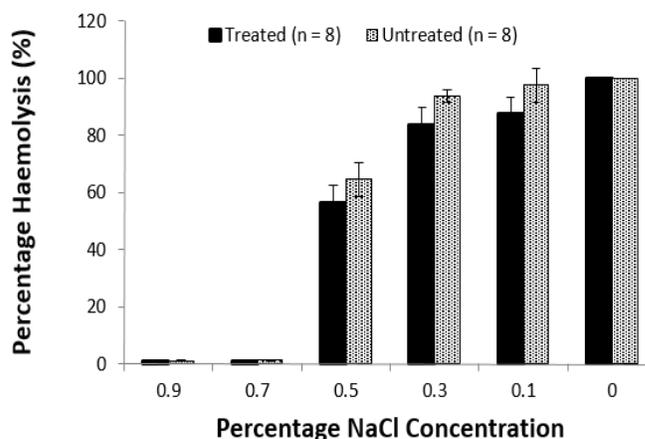
Fig. 2:
Erythrocyte osmotic fragility of horses first week of supplementation with equithrive®. Values are not significantly ($P > 0.05$) different

The total WBC counts recorded were lower in treated horses, decreasing from $9.2 \pm 0.03 \times 10^9$/L to $7.9 \pm 0.4 \times 10^9$/L and $8.41 \pm 0.4 \times 10^9$/L on weeks 3 and 4 of the treatment respectively. The counts were higher in the untreated group increasing from the corresponding counts of $9.1 \pm 0.32 \times 10^9$/L to $9.31 \pm 0.4 \times 10^9$/L and $10.0 \pm 0.4 \times 10^9$/L. The neutrophil counts in treated and untreated group were significantly different. Thus, the neutrophil counts of the untreated group were higher compared to those recorded in the treated horses on weeks 3 and 4 of supplementation, while lymphocyte counts in the untreated and treated group were not different ($P > 0.05$).

The neutrophil/lymphocyte ratio was significantly ($P < 0.05$) greater in the untreated group than treated group on week 3 of the experiment. The oesinophil count on the third week of supplementation with resveratrol was lower than the count recorded pre-administration. There was no difference in the monocyte counts obtained in both treated and untreated horses during the period of supplementation (Table 1)

There was no significant difference between the EOF in control and treated horses on the first week of the experiment. However, on the second week of the experiment, there was an increase in haemolysis in the untreated group at 0.5%, 0.3% and 0.1% NaCl, compared to treated group (Fig. 3). Furthermore, there was a significant ($P < 0.05$) decrease in percentage haemolysis in treated group compared to the control group at 0.3% and 0.1% NaCl on third week of supplementation with resveratrol (Fig. 4). On the fourth week of the experiment, the difference in the percentage haemolysis were not significant ($P > 0.05$) in both groups

Table 1
Changes in haematological parameters in horses administered equithrive® (Mean ± SEM)

|	Week 0		Week 1		Week 2		Week 3		Week 4	
	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated
RBC (x10⁶/µl)	6.0 ± 0.5	6.0 ± 0.5	6.9 ± 0.4	6.0 ± 0.4	6.6 ± 0.3	6.0 ± 0.2	7.5 ± 0.4[a]	6.4 ± 0.2[b]	7.7 ± 0.4	7.2 ± 0.3
Hb (g/dl)	12.0 ± 0.9	12 .4 ± 0.9	13.2 ± 0.6	12.4 ± 0.6	14.6 ± 0.9[a]	12.0 ± 0.7[b]	15.0 ± 0.9[a]	12.8 ± 0.4[b]	5.5 ± 0.7	14.0 ± 0.7
PCV (%)	35.4 ± 2.4	35.0 ± 2.5	39.8 ± 1.7	36.1± 1.4	43.1 ± 2.3[a]	36.0 ± 2.3[b]	45.6 ± 2.3[a]	39.8 ± 1.1[b]	46.5 ± 2.2	43.1 ± 1.8
WBC (x10⁹/L)	8.6 ± 0.5	8.6 ± 0.5	8.7 ± 0.5	9.2 ± 0.3	8.3 ± 0.4	9.1 ± 0.4	7.9 ± 0.4[a]	9.3 ± 0.4[b]	8.4 ± 0.4[a]	10 ± 0.4[b]
NEU (x10⁹/L)	4.7 ± 0.3	4.7 ± 0.3	4.51 ± 0.5	4.85 ± 0.4	4.35 ± 0.4	5.05 ± 0.4	4.19 ± 0.4[a]	5.48 ± 0.4[b]	4.64 ± 0.4[a]	6.0 ± 0.3[b]
LYM (x10⁹/L)	3.9 ± 0.2	3.7 ± 0.2	3.75 ± 0.3	3.85 ± 0.3	3.43 ± 0.2	3.45 ± 0.3	3.2 ± 0.6	3.1 ± 0.1	3.28 ± 0.2	3.35 ± 0.2
N/L ratio	1.2 ± 0.1	1.3 ± 0.1	1.2 ± 0.2	1.2 ± 0.1	1.3 ± 0.1	1.6 ± 0.2	1.3 ± 0.1[a]	1.8 ± 0.1[b]	1.4 ± 0.2	1.8 ± 0.1
EOS (x10⁹/L)	0.55 ± 0.1[a]	0.52 ± 0.1	0.55 ± 0.1	0.54 ± 0.1	0.41 ± 0.1	0.5 ± 0.1	0.38 ± 0.1[b]	0.48 ±0.1	0.5 ± 0.1	0.5 ± 0.0
MON (x10⁹/L)	0.3± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.23 ± 0.1	3 ± 0.1	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.3	0.2 ± 0.1	0.2 ± 0.0

RBC = Red blood cell; Hb = Haemoglobin; PCV = Packed cell volume; WBC = Leucocyte; N/L = Neutrophil/Lymphocyte ratio.
NEU = Neutrophil; LYM = Lymphocyte; EOS = Eosinophil; MON = Monocyte. Values with different letters are significantly different ($P < 0.05$).
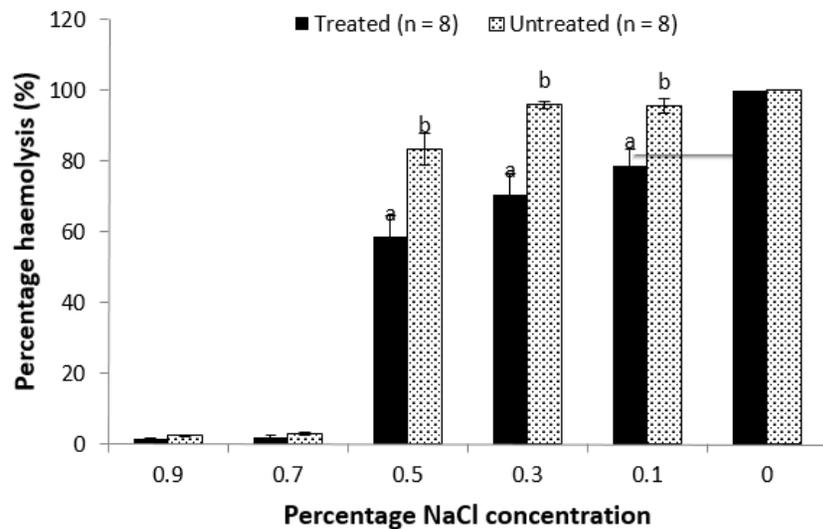
Fig. 3:
Erythrocyte osmotic fragility of horses second week of supplementation with equithrive®. Values with different letters are significantly ($P < 0.05$) different.
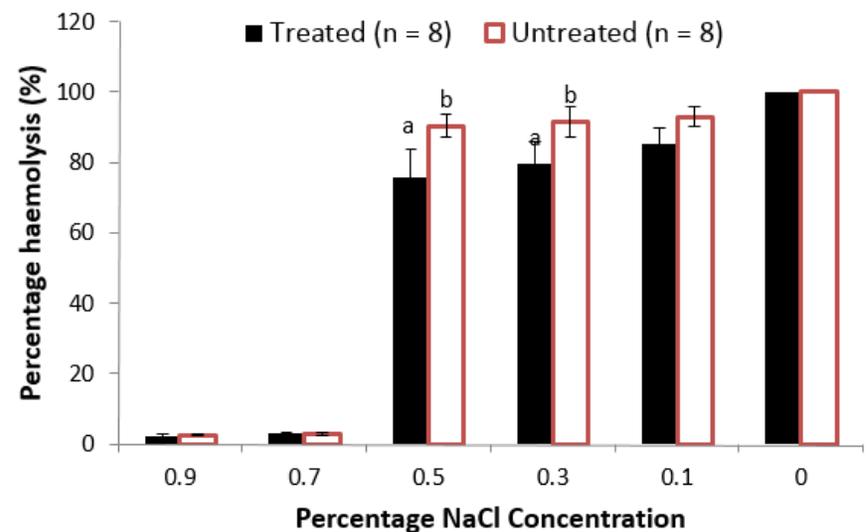
Fig. 4:
Erythrocyte osmotic fragility of horses third week of supplementation with equithrive® Values with different letters are significantly ($P < 0.05$) different.
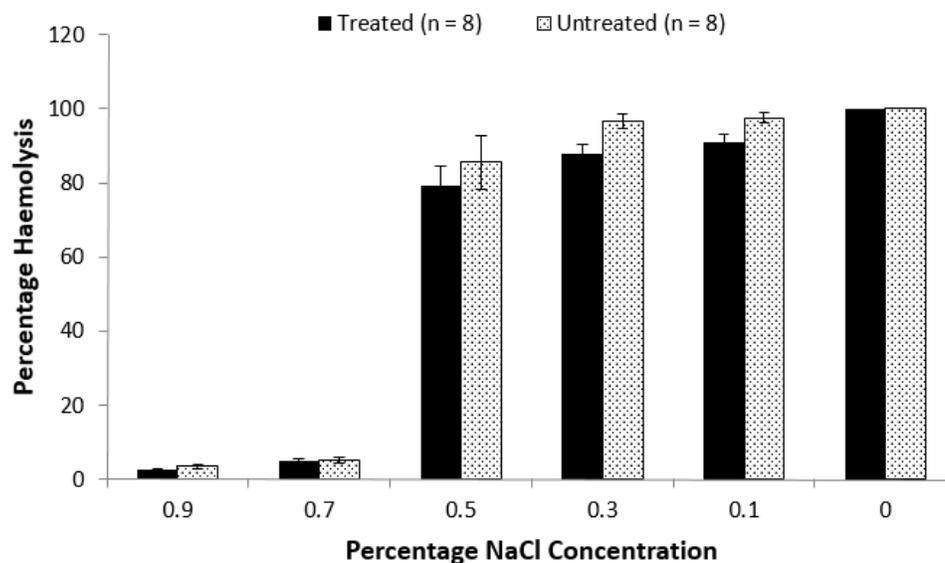
Fig. 5:
Erythrocyte osmotic fragility of horses fourth week of supplementation with equithrive® Values are not significantly (P > 0.05) different.

DISCUSSION

The results obtained from the present study showed an increase in the PCV, haemoglobin concentration and erythrocyte counts, in the treated horses, demonstrating the ability of reveratrol present in the equithrive joint® to maintain the membrane integrity of erythrocyte (Tedesco *et al.,* 2000). Yaqub *et al.* (2014) showed similar result in horses exposed to transport stress and administered with ascorbic acid. This finding also agrees with the result obtained by Atmaca *et al.* (2014) in rats, where resveratrol reduced haemolysis induced by fluoride treatment.

The decrease in white blood cell counts in treated horses shows the anti-inflammatory characteristic of resveratrol (Donnelly *et al.,* 2004). Previous work reported that resveratrol attacks the gene production of cytokines in the immune system that build inflammation which can lead to osteoarthritis (Horokov and Adams, 2008). Studies on suppressive effects of resveratrol on leucocyte count have been reported in rats (Hişmioğullari, *et al.,* 2013) and pigs (Holešovská *et al.,* 2009).

In the present study, a significant decrease in neutrophil count in the treated horses also further demonstrated the anti-inflammatory effect exerted by resveratrol (Lorney *et al.,* 2010). Kohnen *et al.* (2007) reported that resveratrol exerts inhibitory effect on equine neutrophil myeloperoxidase, while Tou and Urbizo (2008) observed the inhibitory effect of resveratrol on degranulation of stimulated human neutrophils. Resveratrol scavenging activity also protects the cells and tissues against oxidative damage, which may be related to reduction of phagocytic response (Kasdallah-Grissa *et al.,* 2007).

The significantly (P < 0.05) decrease in neutrophil/lymphocyte ratio, obtained in treated horses demonstrated that resveratrol administration prevented an increase in oxidative stress in the aged and lame horses. The result agrees with the findings of (Minka and Ayo, 2007) who obtained a decrease in the N/L ratio in goats exposed to transport stress and administered with ascorbic acid. Thus, the decrease in neutrophil/lymphocyte ratio following administration of resveratrol showed that resveratrol is an immune promoter and anti-stress agent. The result of the present study suggests that N/L ratio may serve as an indicator of effects of ageing in horses.

The significant decrease in eosinophil counts on the third week of resveratrol supplementation, compared with pre-administration value showed the ability of resveratrol to reduce the predominant inflammatory cells in allergic reactions. Thus, resveratrol could inhibit eosinophil activation and be beneficial in the prophylactic or symptomatic treatment of allergies in horses.

The significant decrease in EOF recorded in the treated horses revealed the ability of resveratrol present in the equithrive to ameliorate haemolysis associated with ROS, apparently by acting as an antioxidant (Tedesco *et al.,* 2000). The finding agrees with that of Tadolini *et al.* (2000), who reported that resveratrol is a potent free radical scavenger that prevents lipoperoxidation in cytomembranes, and, hence, reduces damage to cell membranes and destruction. Similar result was observed in donkeys (Olaifa *et al.,* 2012) and horses (Yaqub *et al.,* 2014) exposed to packing and transport stress respectively and administered with ascorbic acid, another antioxidant.

The co-administration of sodium hyauronic acid and resveratrol present in equithrive joint® might have contributed to the overall effects observed. Although the results did not provide a clear explanation about the mechanisms by which sodium hyauronic acid potentiated the effect of resveratrol, they suggest that sodium hyaluronic acid and resveratrol worked synergistically to reduce oxidative stress caused by ageing and lameness in horses. Hyaluronic acid has been used in horse where it worked to relieve inflammation of the joint (Kawcak *et al.,* 1997). This may indicate that co-administration of resveratrol and hyaluronic acid may help to reduce EOF in lame and aged horses and may be of value in reducing oxidative stress and their deleterious effects on locomotion in horses.

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