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ORIGINAL ARTICLE

Pycnogenol® supplementation improved the erythrocyte stability of packed donkeys during the late hot-dry season in Zaria, Nigeria

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Abstract
The aim of this study was to determine the effect of pycnogenol® (PYC) on erythrocyte stability of donkeys subjected to packing during the late hot-dry season. Ten donkeys, divided into two groups, served as experimental subjects. P-PYC group ($n = 5$) was subjected to packing only, while P+PYC group ($n = 5$) was administered with PYC (10 mg/kg) and subjected to packing. Erythrocytes obtained from each animal in the experimental groups were added into saline solution of different concentrations (0.9, 0.6, 0.4. 0.2, and 0.0 g/100 ml). Erythrocyte osmotic fragility index (EOFI) was evaluated spectrophotometrically. The concentration of isoprostane was also determined. The mean EOFI was significantly ($P < 0.05$) higher in P-PYC group 3 and 7 days after packing at 0.6 g/100 ml, with values of 32.4 ± 3.2 and $53.4 \pm 4.3\%$, respectively. However, there was no significant ($P > 0.05$) difference in the MCF recorded in both groups, before, after, 3, and 7 days after packing. Percentage erythrocyte stability was significantly higher ($P < 0.05$) in P+PYC group, especially during, and 7 days after packing with values of 24.3 ± 9.2 and $-23.3 \pm 22.8\%$, respectively when compared with the values of 8.6 ± 3.3 and $-79.7 \pm 9.3\%$ obtained in P-PYC group, respectively. Mean isoprostane concentration was significantly ($P < 0.05$) higher in P-PYC group when compared with the value in P+PYC group, 7 days after packing. In conclusion, environmental and packing stresses decreased stability of the erythrocytes in donkeys; however, PYC may have played an ameliorative role.

Keywords Erythrocyte stability · Season · Isoprostane · Donkey

Introduction

Erythrocyte osmotic fragility index (EOFI) measures the resistance of erythrocytes to hemolysis due to stress (Oyewale et al. 2011). It is used to evaluate the level of stability and physiology of erythrocyte cell membrane (Chikezie et al. 2009). When stability of erythrocyte plasma membrane is compromised, some compounds embedded in the membrane are released, including isoprostane which is a marker of lipid peroxidation. Over the last few years, increased plasma levels of reactive oxygen species have been associated with different disease conditions, and among biomarkers of oxidative stress are by-products of lipid peroxidation. Thus, isoprostane is a novel biomarker of lipid peroxidation (Czerska et al. 2016). It is formed when there is a damage to tissue biomolecules especially lipids by free radicals. Thus, it is a peculiar compound formed within the animal system when arachidonic acid undergoes peroxidation (Milne et al. 2011). Isoprostanes are the most specific biomarkers of lipid peroxidation, they are however the most difficult to evaluate (Yoshida et al. 2013). Determination of isoprostane is the most reliable approach in measuring increased production of reactive oxygen species, and its products are potentially active; thus it is involved in the pathophysiology of disease (Miller et al. 1993). However, the use of isoprostane as a biomarker of stress in work donkeys has not been elucidated.

Generally, in the northern region of Nigeria, the late hot-dry season has been reported to be stressful (Igono et al. 1982).

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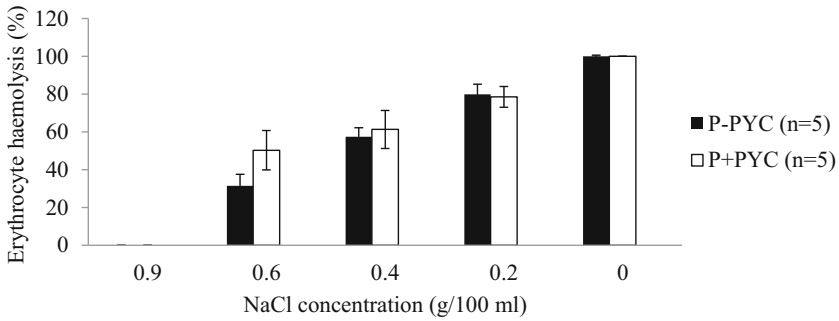

Fig. 1 Effect of pycnogenol® (PYC) on the erythrocyte osmotic fragility of donkeys before packing period

During this season, there is high dry-bulb temperature and high relative humidity and the physiology of the animals are compromised, since they try to adjust to changes in environmental factors (Dzenda et al. 2011). Thus, environmental cues may contribute as stressor to animals such as donkeys. Pycnogenol (PYC), a potent antioxidant which has been used in different species of animals, including guinea pigs (Gunel et al. 2016), dogs (Cerbo et al. 2017), and humans (Rohdewald 2002). It is a supplement extracted from the bark of the French maritime pine tree found commonly in southern France. The active substance contained in PYC is oligomeric proanthocyanidins (OPC). It is pertinent to investigate the ability of PYC to improve the stability of erythrocytes of donkeys subjected to packing. The objective of this study, therefore, was to determine the effect of PYC on erythrocyte stability of donkeys subjected to packing during the late hot-dry season.

Materials and methods

Study area

The study was carried out during the late hot-dry season (May, 2017) at the National Animal Production Research Institute (NAPRI), Shika-Zaria, located in the Northern Guinea Savannah zone of Nigeria (11° 12′ N; 7° 33′ E); with average ambient temperature (AT) of 36.0 ± 0.9 °C, average relative humidity (RH) of 38.0 ± 2.3%, and temperature-humidity index (THI) of 134.8 ± 1.1 during the study period.

Recording of thermal environment conditions

The AT was recorded at the experimental site, using a wet- and dry-bulb thermometer (Brannan, England), and RH was obtained using Osmond's hygrometric table (Narindra Scientific industries, Haryana, India). THI was calculated using the method of Bennett-Wimbush et al. (2014).

Experimental animals and management

Ten, apparently, healthy free-ranging male donkeys weighing 141 ± 2.2 kg were used as experimental subjects. All the animals were identified using ear tags. These donkeys were between the ages of 4 and 5 years. The donkeys were pre-conditioned for 2 weeks, during which they were screened and treated against ecto- and endoparasites. They were kept under semi-intensive management system with maize and sorghum bran supplementation. In addition, 1 kg of whole sorghum grain was fed to each donkey per day. The donkeys were

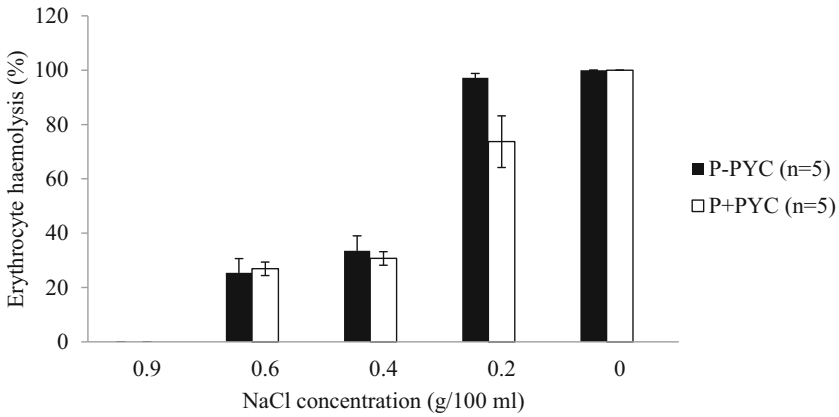

Fig. 2 Effect of pycnogenol® (PYC) on erythrocyte osmotic fragility of donkeys during the packing period

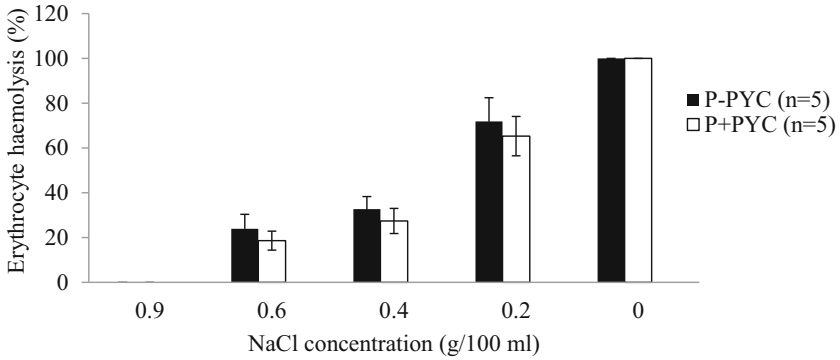

Fig. 3 Effect of pycnogenol® (PYC) on erythrocyte osmotic fragility of donkeys after the packing period

given access to water ad libitum throughout the experimental period.

Experimental design

The test donkeys were first supplemented with antioxidant (PYC) for 5 days. Following this supplementation with PYC, all donkeys were divided into two groups and subjected to packing for 3 days, 1 day apart. P-PYC group (control; $n = 5$) consisted of donkeys with loading but without PYC supplementation, while P+PYC group (test; $n = 5$) consisted of donkeys with loading and PYC supplementation for 5 days (P+PYC). All donkeys in each group were subjected to packing, carrying of load of 50% of their body weight (Legha et al. 2018), and trekking a distance of 10 km according to the method adopted by Olaifa et al. (2018). Blood samples were collected before, during, after, and 3 and 7 days after packing the donkeys.

Administration of PYC

PYC (a registered trademark of Horphag Research Ltd., Geneva, Switzerland) was purchased from Viva Naturals Inc., New York, USA. Group I donkeys received feed only, while group II were fed and administered with PYC orally and individually. The test group received a 5-day multiple ingestion (Grimm et al. 2006) of PYC at a dose of 10 mg/kg mixed in feed (Yang et al. 2008) before subjecting them to exercise trial. PYC was administered to this group in the morning when they were being fed.

Determination of erythrocyte osmotic fragility index (EOFI) and erythrocyte stabilization

The EOFI was determined according to the method described by Oyewale et al. (2011). The quotient of absorbance of the content of each test tube (1st–5th) and the 6th test tube was multiplied by a factor of 100. The ranges of values represent the percentage of hemolysis at each corresponding PBS concentration (0.9–0.1 g/100 ml). Mean corpuscular fragility (MCF) index was determined using the corresponding concentration of saline solution that caused 50% lysis of red blood cells (Krogmeier et al. 1993). The cumulative erythrocyte osmotic curve, the plot of percentage hemolysis versus concentrations of PBS solution was used to obtain the MCF values. Relative stability was calculated according to a formula by Chikezie (2007):

$$\text{Relative stability } (\%) = \text{MCF}_{\text{control}} - \text{MCF}_{\text{test}} / \text{MCF}_{\text{control}} \times 100$$

Determination of isoprostane concentration

The isoprostane concentration was determined using enzyme immunoassay technique (Roberts II and Morrow 2000). The

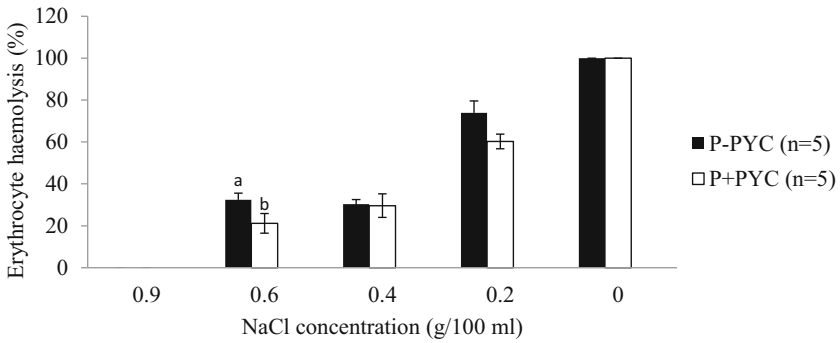

Fig. 4 Effect of pycnogenol® (PYC) on erythrocyte osmotic fragility of donkeys 3 days after packing period

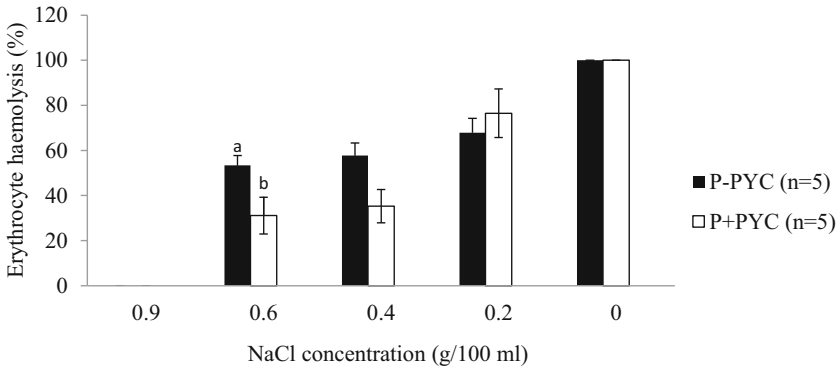

Fig. 5 Effects of pycnogenol® (PYC) on erythrocyte osmotic fragility of donkeys 7 days after packing

assay with the commercial kit, the donkey kit (My Biosource Inc., San Diego, US) is a competitive enzyme-linked immunosorbent assay (ELISA) for determining levels of 8-isops F2t (the best characterized isoprostane) in plasma samples. Briefly, plasma samples were mixed with an enhancing reagent that essentially eliminates interferences due to non-specific binding. The 8-isops F2t in the samples or standards competes with 8-isops F2t conjugated to horseradish peroxidase for binding to a polyclonal antibody specific for 8-isops F2t coated on the microplate. The horseradish peroxidase activity results in color development when substrate is added, with the intensity of the color proportional to the amount of 8-isops F2t-horseradish peroxidase bound, and inversely proportional to the amount of unconjugated 8-isops F2t in the samples or standards.

Statistical analyses

Statistical differences between and within groups were analyzed by a one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison *post hoc* test. Data were expressed as mean ± standard error of the mean (mean ± SEM). GraphPad Prism 5.3 for Windows (GraphPad Software, San Diego, California, USA) was used for the analyses. Values of $P < 0.05$ were considered significant (Snedecor and Cochran 1994).

Results

Figures 1, 2, 3, 4, and 5 show the effect of PYC on EOFI of donkeys before, during, after and 3 and 7 days after packing. The values of percentage hemolysis obtained in P-PYC group at 0.6 g/100 ml (32.4 ± 3.2 and 53.4 ± 4.3%) were significantly ($P < 0.05$) higher than the values of 29.6 ± 5.6 and 35.3 ± 7.4%, 3 and 7 days after packing, respectively.

Figure 6 shows MCF of erythrocyte of donkeys administered with PYC and subjected to packing during the late hot-dry season. Fifty percent of erythrocytes were hemolyzed before, during, after, and 3 and 7 days after packing at concentrations of 0.4 ± 0.2 g/100 ml, 0.4 ± 0.01 g/100 ml, 0.3 ± 0.03 g/100 ml, 0.3 ± 0.1 g/100 ml, and 0.5 ± 0.1 g/100 ml, respectively in P-PYC groups. In P+PYC group, the corresponding MCF occurred at the concentrations of 0.5 ± 0.1 g/100 ml, 0.3 ± 0.03 g/100 ml, 0.3 ± 0.03 g/100 ml, 0.3 ± 0.1 g/100 ml, and 0.4 ± 0.1 g/100 ml.

Table 1 shows the stability of erythrocytes donkeys supplemented with PYC and subjected to packing during the late hot-dry season. An increase in erythrocyte stability was recorded in P+PYC group when compared with that of P-PYC group, especially during packing (8.6 ± 3.3% versus 24.3 ± 9.2%, respectively) and 7 days post-packing (− 79.7 ± 9.3% versus − 23.3 ± 22.8%, respectively).

Figure 7 shows the concentration of isoprostane in donkeys supplemented with PYC and subjected to packing during the late hot-dry season. There was a significant ($P < 0.05$) decrease in the level of isoprostane in the P+PYC group with

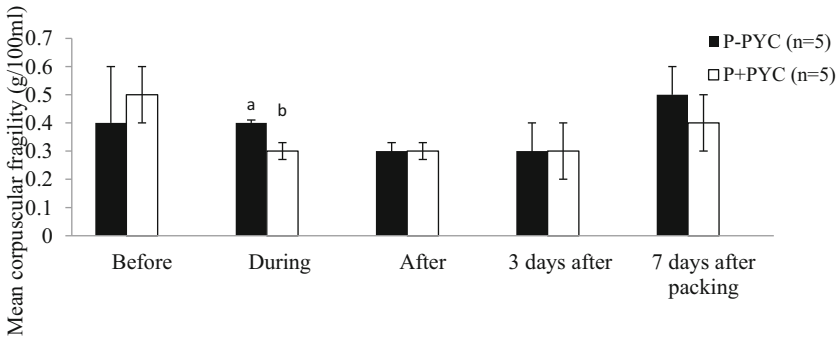

Fig. 6 Mean corpuscular fragility of erythrocyte of donkeys supplemented with pycnogenol® (PYC) and subjected to packing during the late hot-dry season

Table 1 Stability of erythrocytes in packed donkeys administered with pycnogenol® (PYC) during the late hot-dry season

Packing period	P-PYC ($n = 5$, %)	P + PYC ($n = 5$, %)
Before packing	− 50.8 ± 8.8	− 42.9 ± 5.1
During packing	8.6 ± 3.3[a]	24.3 ± 9.2[b]
After packing	6.6 ± 11.3	8.6 ± 12.4
3 days post-packing	4.2 ± 17.7	7.5 ± 9.3
7-days post-packing	− 79.7 ± 9.3[a]	− 23.3 ± 22.8[b]

Values with different superscripts[a,b] on the same row are significantly different

value of 382.8 ± 64.7 pg/ml, when compared with the value of 671.6 ± 44.4 pg/ml in P-PYC group, 7 days after packing.

Discussion

The THI obtained during the study was higher than the maximum value of 80 reported to be optimum in unstressed donkeys. This, therefore, indicates that the pack donkeys were subjected to the concomitant effects of stress due to both heat and packing stresses during the late hot-dry season.The higher percentage haemolysis observed in P-PYC group indicates that the erythrocytes were less resistant to hemolysis in hypotonic solution. Although, the donkeys in the two groups were exposed to similar conditions, it appears that the P-PYC group may have been more stressed than P+PYC group who were administered with PYC. Increased hemolysis may be due to increased sensitivity of erythrocyte membranes to hydrogen peroxide, apparently, released and contributed to oxidative stress, which plays a key role in the pathogenesis of many diseases (Signolet et al. 2016). PYC has been known to be a potent antioxidant; thus, PYC may have acted by stabilizing plasma membrane of red blood cell in P+PYC group by improving nitric oxide production, which leads to vasorelaxation, thereby improving blood flow. There was no significant difference between the MCF of the control and test groups before, after, and 3 days after packing; however, the MCF in P+PYC group during packing occurred at a significantly lower concentration when compared to the P-PYC group. Thus, it can be deduced that the erythrocytes of the control donkeys were more fragile especially at significantly higher concentrations during and probably 7 days after packing. It could be that PYC enhanced antioxidant effect, particularly during and 7 days after packing. The erythrocytes of P+PYC group were more stable when compared with P-PYC group, especially during packing and 7 days post-packing. This denoted that in the P+PYC group, the erythrocyte plasma membrane was more stable. This may be as a result of the oral supplementation with PYC, which has been known to have antioxidative properties and is 50 times more potent than vitamin C, and 25 times more potent than vitamin E. This finding agreed with the report of Lee et al. (2013), who observed that PYC increases the activity of antioxidant enzymes, thereby improving the stability of red blood cells.

Isoprostane is one of the products of lipid peroxidation among others like MDA. However, it is highly specific, because it is embedded within the phospholipid, situated within the structural plasma membrane of tissues (Yoshida et al. 2013). Thus, its use as a biomarker indicates that plasma membrane of cells must have been compromised. The increased production of isoprostane in both groups before packing suggests that even though they were not yet packed, some external cues such as thermal environment parameters might have played a role in inducing oxidative stress. The increased production of isoprostane, especially 7 days after packing following subjection to packing and exposure to other environmental cues, may have been mitigated by PYC. The mitigation was evident by the reduction in isoprostane concentration in P+PYC group, thus played a major role in preventing the increased production of isoprostane over a long period of time after packing. Isoprostanes are like prostaglandin compounds derived from lipid peroxidation, consequent to the effect of reactive oxygen species. It has been reported that isoprostanes are also released during exercise (Braunwald 2008). Their increase during exercise reflects oxidative damage to plasma membranes following muscle activity (Margonis et al. 2007).

In conclusion, the administration of PYC enhanced the stability of the donkey erythrocyte and reduced lipid peroxidation, the effect of which could be sustained for a relatively long period (2 weeks) after exposure to stress. Thus, donkey

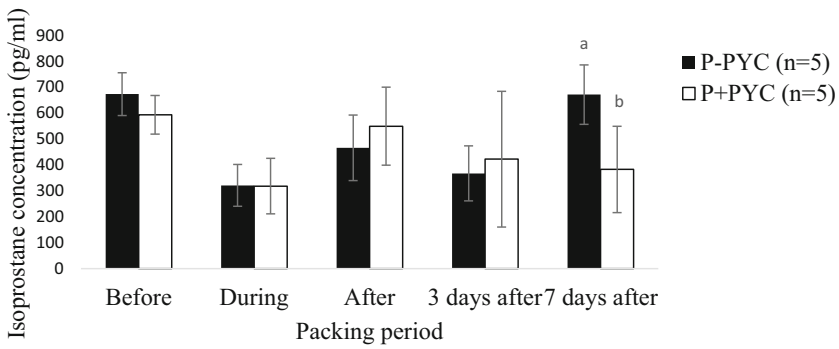

Fig. 7 Effect of isoprostane concentration in donkeys administered with pycnogenol® (PYC) and subjected to packing

owners could make use of this supplement some days before packing to ensure maximal health and good performance, thereby improving the welfare of donkeys used for packing.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The animal experiments within this study were approved by the Institutional Animal Care and Use Committee of Ahmadu Bello University, Zaria, Nigeria and were performed in accordance with the animal welfare guidelines thereof.

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