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Modulatory Effects of Resveratrol Supplementation on Inflammatory Markers in Ageing and Lame Horses

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Abstract: The study was carried out to evaluate the effects of equine resveratrol supplement (equithrive joint™) on inflammatory indices in ageing and lame horses. 16 horses of both sexes aged 18±0.65 years, showing lameness grade 3 were used for the study. The horses weighed 350-450 kg and were randomly assigned to treated and untreated groups comprising 8 horses each. Treated group were administered with resveratrol supplement for 4 weeks while the untreated group were given only *Saccharomyces cerevisiae* yeast strain used as carrier in the supplement**.** Blood samples were collected from each animal before supplementation on week 0 and then weekly for four weeks of supplementation. Markers of inflammation consisting of tumour necrosis factor-alpha (TNFα), erythrocyte sedimentation rate (ESR), haematocrit level and serum protein concentration were determined by standard methods. Equithrive joint ™ supplementation reduced significantly ($p<0.05$) the values of TNF-α concentration, ESR and serum protein in the treated horses compare with the controls. The haematocrit level was significantly ($p<0.05$) higher in treated horses compared with the controls. It was concluded that equithrive joint ™ is a potent anti-inflammatory agent capable of reducing inflammatory mediators and thereby enabling horses move with ease particularly during ageing.

Keywords: Erythrocyte sedimentation rate, horse, inflammation, resveratrol, tumour necrosis factor-alpha

INTRODUCTION

Ageing in horses causes stiffening of joints and inability to meet the same performance standards that they did while younger (Johnson *et al*., 2013). Geriatric horses (15 years and above) are exposed to harmful effects of chronic inflammation, leading to loss in vitality and diminishing quality of life (Lawan *et al*., 2013). Old horses have increased production of inflammatory compounds as they suffer more from self-inflicted trauma during turn-out, laminitis, age-related tissue break-down, cancer, heart problems and obesity (Watson, 2006). Prolonged inflammation leads to a progressive shift in the type of cells present at the site of inflammation and is characterized by simultaneous destruction and healing of the tissue from the inflammatory process (Eming *et al*., 2007). Measurement of inflammatory markers has two main functions: to detect acute inflammation that may indicate specific diseases and to give a marker of treatment response (Hamilton *et al*., 2012).

Preliminary evidence suggested that evaluating blood levels of certain inflammatory markers might indicate a horse that is at increased risk of developing laminitis (Al-Derawie, 2012; Wray *et al*., 2013).

Tumour necrosis factor alpha (TNFα) is a pleiotropic cytokine with many different effects including triggering the production and secretion of pro-inflammatory mediators and being cytotoxic against various tumor cells and induction of apoptosis of various cells (Popa *et al*., 2007; Navarro *et al*., 2012). TNF-α is also associated with chronic low-grade inflammation (Maachi *et al*., 2004) and responsible for the cardinal clinical designations of inflammation including heat, redness, swelling and pain (Drake, 2007).

Erythrocyte sedimentation rate (ESR) is a simple non-specific screening test that indirectly measures the presence of inflammation in the body (Sox and Liang, 1986). It reflects the tendency of red blood cells to settle more rapidly in the face of some disease states (Liu *et al*., 2013). Alteration in the distribution of charges on the surface of the red blood cell that normally keeps them separate from each other results in their forming aggregates known as rouleaux (ICSH, 1993). ESR also reflects changes in the plasma proteins that accompany acute and chronic infections. Several studies investigated diagnostic values of ESR in inflammatory disease and established that ESR is a potential biomarker for disease differentiation (McGregor, 2013), Most horse trainers monitor ESR as guide to check health status of their horses (Rashid, 1997). A reduction in haematocrit level is associated with increase in ESR and plasma protein (Allen, 1988).

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When the haematocrit level reduces, the velocity of the upward flow of plasma is altered so that red blood cell aggregates fall faster resulting in high ESR (Holley *et al*., 1999).

There are many feed supplements available, which are widely used to aid joint repair and reduce stiffness (Duren, 2005). In the field, trainers and horse owners have noticed an improvement in health, comfort and performance level in horses, receiving equine resveratrol (Equithrive ^{TM}) therapy (P. Lawless Biological Prospects, University of Kentucky, Personal communication). (Resveratrol (3,5,4'-trihydroxystilbene) is a polyphenol, found naturally in a variety of sources, including grapes, Japanese knotweed, berries, peanuts, dark chocolate and tea (Shishodia and Aggarwal, 2006). Resveratrol has received research attention for its anti-inflammatory effects (de la Lastra and Villegas, 2005; Baur and Sinclair, 2006). It has a lot of health benefits, including prevention of joint diseases and improvement of athletic endurance (Opie and Lecour, 2007; Zahedi *et al*., 2013). It exerts anticancer, antimicrobial, anti-ageing and antioxidant effects (Aggarwal and Harikumar, 2008). Equithrive^{TM} also contains sodium hyaluronic acid which aids to cushion and reduce pain in the joint (Puhl and Scharf, 1997). Hyaluronic acid is used in horses as an intravenous injection, where it works systemically to relieve inflammation of the joint (Kawcak *et al*., 1997). Previous work by Ialenti and DiRosa (1994) showed that both acute and chronic inflammations were resolved by hyaluronic acid. It is used for oral drug delivery systems because it is highly compatible with the tissues of the oral mucosa, which contains large amounts of hyaluronic acid (Andrews *et al*., 2009).

The study was aimed at evaluating the effects of equithrive joint^{TM} on inflammatory indices in ageing and lame horses.

MATERIALS AND METHODS

Animals and blood sampling: The experiment was carried out in a polo farm in Kaduna (10º29′N, 07º28′E), located in the Northern Guinea Savannah zone of Nigeria. The study involved 16 horses of both sexes, aged 18 ± 0.65 years, weighing between 350-450 kg and showing lameness grade 3 (Stashak, 1987) due to arthritis of the knee, hock, stifle and fetlock joints. They were randomly assigned to treated and untreated (control) groups of eight animals each. The horses were housed in standard horse stables measuring 10×12 m made of concrete floor, cement block wall and asbestos roof and well ventilated. They were fed with wheat bran, sorghum, hay and fresh pasture. The experimental horses were pre-conditioned for two weeks before the commencement of the supplementation; during this period, they were assigned to experimental stables and treated for ectoparasites and endoparasite.

Resveratrol supplement (Equithrivejoint^{TM}) was purchased from Hagyard Pharmacy, Kentucky, USA. Treated horses were fed 30 g of equithrive joint^{TM} powder containing 2 g of resveratrol and 200 mg sodium hyaluronic acid and the carrier *Saccharomyces cerevisiae* as loading dose for the first ten days of the experiment and then 15g of equithrive joint^{TM} powder containing 1g of resveratrol and 100 mg sodium hyaluronic acid and the carrier, *Saccharomyces cerevisiae* as maintenance dose for the remaining 18 days of the study. Untreated horses were fed 30g of the carrier *Saccharomyces cerevisiae* as loading dose for the first ten days of the experiment and then 15 g of the carrier, *Saccharomyces cerevisiae* as maintenance dose for the remaining 18 days of the study. The supplement was mixed in their daily feed during the period of the study (D. Horokov and A. Adams, University of Kentucky, Gluck Equine Research Center, Personal communication). The two 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 water provided *ad libitum.*

Blood sample collection: Blood samples were collected from each animal in the morning before feeding. This was done before supplementation on week 0 and then at first, second, third and fourth week of supplementation. At each period of blood sampling, 7 mL of blood was collected by jugular venipuncture of each horse using disposable syringes and 18 gauge×1.5" sterile needles. 2 mL of the blood was placed in tubes containing 0.5 mL sodium citrate and was used to measure erythrocyte sedimentation rate. Another 2 mL of the collected blood was also placed in tubes containing ethylenediaminetetra acetic acid (EDTA) to determine the haematocrit level. The remaining 3 mL was put in a sterile bottle without anticoagulant, placed in ice and allowed to clot for 30 min before centrifugation for 15 min at approximately 1000×g. The resultant serum was removed immediately and placed in plain tubes for analyses of TNF-α concentration and serum protein; the samples were stored at -20°C until day of analysis.

Laboratory analysis: Equine tumour necrosis factor-alpha (TNFα) kit was purchased from USCN Life Science Inc, Houston, Texas, USA. The test principle applies a Sandwich enzyme immunoassay. The microtiter plate provided in the kit has been pre-coated with an antibody specific to TNFα. A 100 µL standard or sample was added to the appropriate microtitre plate wells with a biotin-conjugated antibody specific to TNF-α and then incubated at 37ºC for 2 h. Thereafter,

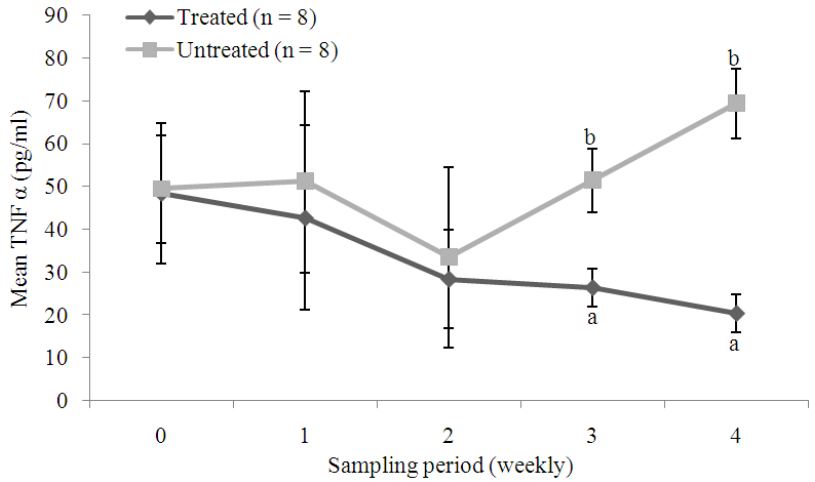

Fig. 1: The TNF-α serum concentration (pg / mL) in horses administered with equithrive joint^{TM} for four weeks (Mean±SEM), Values with different alphabets are statistically (P < 0.05) significant

avidin conjugated to horseradish peroxidase was added to each microplate well and incubated at 37ºC for 1 h. Then Tetramethylbenzidine (TMB) substrate solution was added. Only those wells that contain TNFα, biotin-conjugated antibody and enzyme-conjugated avidin exhibited a change in color. The enzyme-substrate reaction was terminated by the addition of sulphuric acid solution and the colour change measured spectrophotometrically at a wavelength of 450 ± 10 nm. The concentration of TNFα in the samples was then determined by comparing the optical density of the samples to the standard curve (Cloud Clone Corp, 2013).

The Westergren method was used to determine the ESR. Briefly, 2 mL of venous blood collected was placed into a tube containing 0.5 mL of sodium citrate, stored for 2 h at room temperature (25ºC) or 6 h at 4°C. The blood was then drawn into a Westergren-Katz tube to the 200 mm mark. The tube was placed in a rack in a strictly vertical position for 1 hour at room temperature and then the distance from the lowest point of the surface meniscus to the upper limit of the erythrocyte sediment was measured. The distance of fall of erythrocytes, expressed as millimeters in 1 hour was obtained as the ESR (ICSH, 1993).

The blood containing EDTA was used to obtain the haematocrit value as described by Dawies and Lewis (1991) while serum protein was determined via the biuret method as described by Cheesbrough (1991).

Data analysis: 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 TNFα serum concentration showed a significant ($p<0.05$) reduction in the treated compared with the untreated group on the third and fourth weeks of administration of the supplement (Fig. 1). Thus, the TNFα concentration of the treated group was 20.4±4.5 pg/mL and 20.38±4.5 pg/mL on third and fourth weeks of the study while that of the corresponding untreated group was 51.4±7.4 pg/ml and 69.36±8.1 pg/mL. The TNFα concentration values also observed on second, third and fourth weeks of treatment with equithrive joint^{TM} were significantly ($p<0.05$) lower than the pre-administration value.

The ESR base-line values observed from treated and control horses before the study were 49.8 ± 6.4 mm/hr and 50.3±7.0 mm/hr respectively. Following the administration of resveratrol supplement, the ESR value reduced significantly ($p<0.05$) from pre-administration value of 49.8±6.4 mm/h to 26.7±7.2 mm/h and 38.1±9.40 on second and third week, respectively. There was also a significant ($p<0.05$) reduction on the ESR of the treated group compared with the untreated group during the four weeks of the study (Fig. 2).

The concentration of serum protein showed a significant ($p<0.05$) difference between the treated and untreated group on the fourth week of supplementation with equithrive joint^{TM}. Thus, the serum protein was lower in the treated group compared with the untreated group at this period of the experiment (Fig. 3).

The haematocrit values rose from pre-administration value of 35.4±2.42% to 43.1±2.3% and 46.0±2.3% on the second and third weeks of

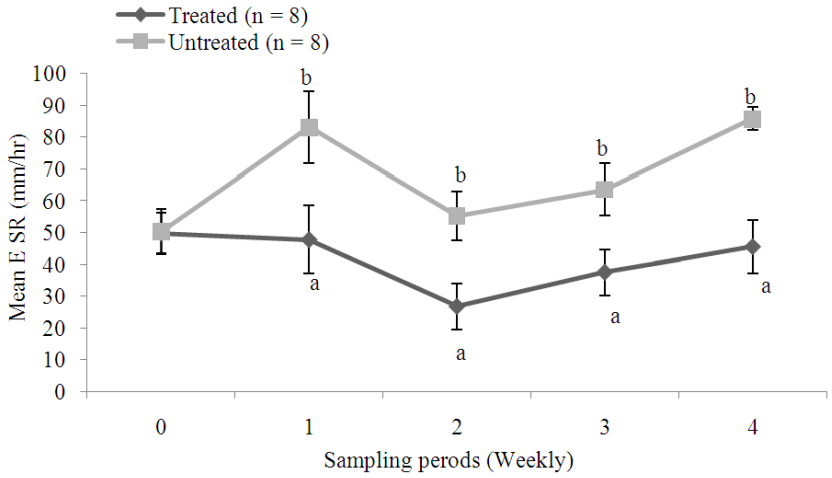

Fig. 2: The ESR (mm/hr.) in horses administered with equithrive joint™ for four weeks (Mean ± SEM). Values with different alphabets are statistically (P < 0.05) significant

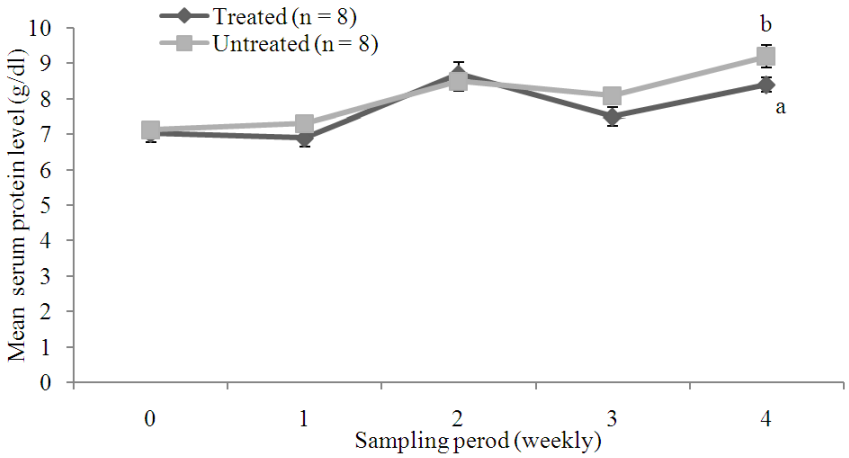

Fig. 3: The Serum protein (g/dl) concentration in horses administered with equithrive joint™ for four weeks (Mean ± SEM), Values with different alphabets are statistically (P < 0.05) significant

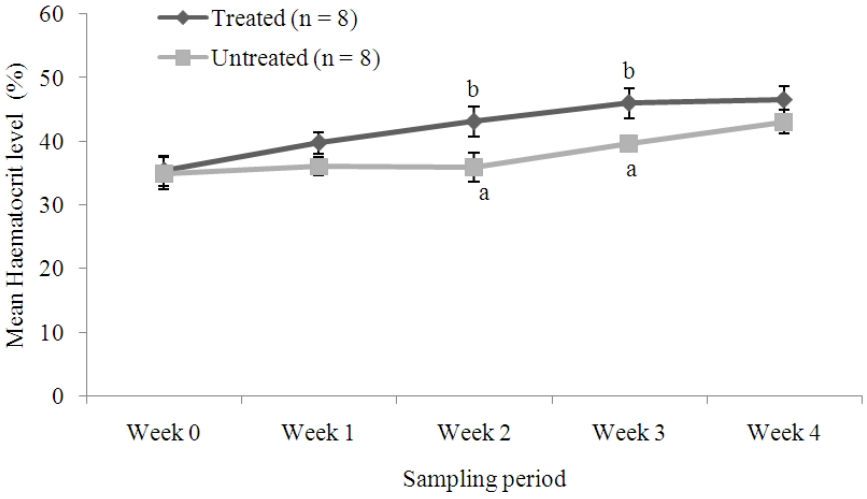

Fig. 4: The Haematocrit (%) in horses administered with equithrive joint™ for four weeks (Mean ± SEM), Values with different alphabets are statistically (P < 0.05) significant

supplementation (Fig. 4). The values were higher ($p<0.05$) than the corresponding values of 35.0±2.5% to 36.0±2.3% and 39.8±1.1% recorded in the untreated horses.

DISCUSSION

The finding by Watson (2006) that inflammation increases with age was shown by the pre-administration values of ESR in both treated and control horses. Thus, the mean ESR values obtained before supplementation were higher than the normal range which is 30-40 mm/hr in horses (Smith, 1996). The Results of Al-Derawie (2012) indicated significant increase in ESR in horses affected with acute laminitis. The report by Oikawa *et al*. (2004) on stress responses to road transport in thoroughbred horses having respiratory disease showed an increase in ESR values, indicating that increase in ESR is a good index of stress response. The fact that the ESR values in the treated horses reduced significantly ($p < 0.05$), compared with that of the untreated horses during the experiment shows that equithrive joint™ is a potent anti-inflammatory agent and this may be due to synergistic effects of resveratrol and hyaluronic acid which are anti-inflammatory and anti-oxidative agents. Lorney *et al*. (2010) also showed that resveratrol is an anti-inflammatory agent while Ialenti and DiRosa (1994) observed that hyaluronic acid is also an anti-inflammatory compound. The reduction in the serum protein concentration in the treated horses demonstrates that equithrive joint™ may exert an ameliorative effect on inflammation. Thus, higher levels may indicate inflammation, chronic infection and certain cancers (Kohnke, 2009).The increase in haematocrit level agrees with work of Allen (1988) who observed an association between decreases in ESR with increase in haematocrit level. This further reveals that equithrive joint™ may help to reduce inflammation due to ageing and lameness.

Measurement of TNF-α by ELISA method has been widely used in clinical investigations and research (Yucel and Aybay, 2005). TNF-α plays role in the pathogenesis or progression of many diseases (Tracey and Ceramy, 1993; Aggarwal and Natarajan, 1996). A decrease in TNF-α concentration observed in the treated horses on the third and fourth weeks of administration of equithrive joint™ agrees with the finding of (D. Horohov and A. Adams University of Kentucky, Gluck Equine Research Center, Unpublished data) who observed a reduction on TNF-α concentration after supplementation of equithrive joint™ to aged horses. The anti-inflammatory activity of resveratrol in this study agrees with the result of Zahedi *et al*. (2013), who demonstrated that resveratrol reduced the plasma levels of TNF-α in male professional basketball players given 200 mg resveratrol for six weeks. The preliminary research carried out by Adam (2013) also exhibited anti-inflammatory activity of resveratrol, based on the findings in equine cytokine production in cell culture.

Co-administration of resveratrol and hyaluronic acid might have reduced the inflammatory markers observed in this study. Kawcak *et al*. (1997) similarly observed that hyaluronic acid used in horse relieved inflammation of the joints while Bergin *et al*. (2006) showed an improvement in lameness conditions in horses receiving oral sodium hyaluronic acid.

CONCLUSION

The ameliorative effects of equithrive joint™ on aged and lame horses were observed as evidenced by the reduction in the inflammatory markers evaluated. Administration of equithrive joint™ may be a good agent in modulating arthritic conditions commonly seen in ageing horses.

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