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Original Research

Ameliorative Effects of Resveratrol on Oxidative Stress Biomarkers in Horses

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ABSTRACT

The aim of the present study was to evaluate the influence of age and lameness on antioxidant status in horses administered resveratrol supplement (Equithrive Joint). A total of 16 horses of both sexes, aged between 15 and 22 years, showing lameness score of 3 and weighing 350–450 kg were used, comprising eight horses which were administered resveratrol supplement for 4 weeks and eight others which served as control and given only *Saccharomyces cerevisiae* yeast strain used as carrier in the supplement. Blood samples were collected from each horse before supplementation (week 0) and at first, second, third, and fourth weeks of the experiment. Serum antioxidant marker of malondialdehyde (MDA) concentration, superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase activities were determined by standard methods. Resveratrol supplement administration reduced significantly ($P < .05$) the concentration of MDA and activity of GPx but increased that of SOD and catalase. The result showed that aging and lameness increased oxidative damage in horses, and resveratrol supplement exerted some protective effects on the aged and lame horses by increasing the antioxidant capacity of the animals.

1. Introduction

Oxidative stress is a harmful imbalance in the oxidative–antioxidative system of cells [1]. Studies have shown that one or more antioxidant enzymes decrease as a consequence of aging [2–4]. Thus, superoxide dismutase (SOD) decreases, whereas catalase and glutathione peroxidase (GPx) increase during aging [5]. Chronic rheumatic disease and degenerative bone and joint diseases are linked to excessive reactive oxygen species (ROS) production [6], which are capable of degrading components of the joint as indicated in the pathogenesis of equine joint disease [7]. Superoxide dismutase, catalase, and GPx are antioxidant enzymes in mammalian cells which are necessary for oxygen metabolism [7]. The SODs convert superoxide radical into hydrogen peroxide and molecular oxygen (O_2), whereas the GPx and catalase convert hydrogen peroxide into oxygen and water [8]. However, the activities of these enzymes may decrease during oxidative stress [9,10]. Lipid peroxidation generates a variety of relatively stable decomposition end products, mainly α, β-unsaturated reactive aldehydes, such as malondialdehyde (MDA), and isoprostanes [11–13], which can then be measured as an indirect index of oxidative stress. Reported results on oxidative stress in horses in the literature were mainly based on the exercise physiology [14–16]. Antioxidant therapies are now designed to prevent oxidative stress which could potentially have significant impact on age-related diseases [17]. Older horses move more slowly and their joint, particularly limbs, are stiffer [18]. Arthritis is common condition and major factor in reducing an older horse's mobility and enthusiasm for exercise and can lead to the early retirement of otherwise healthy animals [19]. Hence, older exercising horses need more antioxidants

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because exercise can intensify their vulnerability to ROS damage [20]. Consequently, there is great interest in evaluating antioxidant agents that could protect aging horses from ROS. One possible agent, resveratrol, is a natural phytoalexin with antioxidant properties [21].

Resveratrol (3,4,5-trihydroxystilbene) is from a class of polyphenolic compounds called stilbenes [22]. It is found in the skins of certain red grapes, peanuts, blueberries, some pines, and the roots and stalks of Japanese knotweed [23]. Resveratrol has a lot of health benefits, including the improvement of cardiovascular and anti-inflammatory properties, prevention of joint diseases, and improvement of athletic endurance [24]. It exerts anticancer, antimicrobial, antiaging, and antioxidant effects [25,26]. This biological activity is carried out by a wide variety of mechanisms, one of the most important of which is antioxidant activity due to its free radical scavenging [27]. Equithrive Joint, a resveratrol preparation for horses, contains a high-quality source of resveratrol which is easily administered PO [28]. It also contains sodium hyaluronic which is used as a viscosupplement and increases the viscosity of the synovial fluid [29] which helps to lubricate, cushion, and reduce pain in the joint [30].

This study was carried out to evaluate the effects of resveratrol on some oxidative biomarkers in aging and lame horses.

2. Materials and Methods

The experiment was carried out in a private polo farm in Kaduna (10°29′N, 07°28′E), located in the Northern Guinea Savannah zone of Nigeria. The study involved 16 horses aged 18.5 ± 0.65 years and showing grade 3 lameness (lameness consistently observed at a trot in all circumstances) [31]. The horses were of different sexes, weighing between 350 and 450 kg. Estimation of their ages was carried out as described by Wayne and Melvin [32] and then confirmed by farm records. They were randomly assigned to treated and untreated (control) groups of eight animals each. They were housed in standard horse stables measuring 10 × 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 preconditioned for 2 weeks before the commencement of the supplementation; and during this period, they were screened and treated for endoparasites and hemoparasites.

The resveratrol supplement was purchased from Hagyard Pharmacy, Lexington, KY. Treated horses were fed four scoops (30 g) of Equithrive Joint powder containing 2,000 mg of resveratrol and 200 mg of sodium hyaluronic acid and the carrier *Saccharomyces cerevisiae* as the loading dose for the first 10 days of the experiment, and then two scoops (15 g) of Equithrive Joint powder containing 1,000 mg of resveratrol and 100 mg of sodium hyaluronic acid and the carrier *S. cerevisiae* as maintenance dose for the remaining 18 days of the study. Untreated horses were fed 30 g of the carrier *S. cerevisiae* as loading dose for the first 10 days of the experiment and then 15 g of the carrier *S. 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 [28]. 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.

2.1. Blood Sample Collection

This was carried out during the 2-week preconditioning period to obtain baseline data and then during the 4-week treatment. Blood samples were collected from each animal in the morning before feeding at weekly intervals. At each blood sampling of each horse, 5 mL of blood was collected by jugular venipuncture using disposable syringes and 18-ga × 1.5-inch sterile needles. The collected blood was poured into a sterile sample bottle without anticoagulant and placed in ice. The samples were allowed to clot for 30 minutes and then centrifuged for 15 minutes at approximately 1,000*g*. The resultant serum was removed immediately and placed in plain sterile tubes and then stored at −80°C for determination of MDA concentration and GPx, SOD, and catalase activities.

2.2. Analysis of Oxidative Stress Biomarkers

2.2.1. SOD Activity

Standard protocol of the Northwest Life Science Specialist, Vancouver, Canada, was used to measure the SOD activity. The method was based on monitoring the autoxidation rate of hematoxylin as originally described by Martin et al [33] with modification to increase robustness and reliability. Briefly, 230 μL of assay buffer was added to wells of the microplate. Then, 10 μL of assay buffer (for blank) and 10 μL of sample were added. The wells were properly shaken, mixed, and incubated for 2 minutes. A multichannel pipette was used to add 10 μL of hematoxylin reagent to begin the reaction. The content of each well was quickly mixed using the instrument's shaker function, and immediately, the absorbance at 560 nm was recorded. The SOD activity was calculated as: SOD U/mL = 1.25 × % inhibition.

2.2.2. GPx Activity

Standard protocol of the Northwest Life Science Specialist, Vancouver, Canada, was used to measure the GPx activity. For standard procedure for microplate assay, all reagents were brought to room temperature (25°C). Diluted sample (50 μL) was added to wells and then 50 μL of working nicotinamide adenine dinucleotide phosphate (NADPH) added to each well. Working H_2O_2 (50 μL) was also added to each well. After waiting for 1 minute, microplate was placed in plate reader and read at 340 nm measurements [34].

To calculate GPx concentration using the NADPH absorption coefficient: the GPx concentration, expressed as mU/mL, was calculated using the GPx activity definition.

$$[GPx] = \frac{2(mRate_s - mRate_b) \cdot V_{R_{xm}}}{2.74 \cdot V_s} \cdot df$$

where $mRate_s = -1{,}000 \times \Delta A340/min$ of sample; $mRate_b = -1{,}000 \times \Delta A340/min$ of blank; 2.74 = NADPH 340 nm millimolar absorption coefficient at 1 cm path length; $V_{R_{xm}}$ = volume of reaction mixture; V_s = volume of sample; 2 = correction for 2 moles reduced glutathione oxidized to 1 mole glutathione disulfide (Oxidized glutathione) per mole NADPH oxidized; df = sample dilution factor.

2.2.3. Catalase Activity

Catalase kit was purchased from Abcam PLC, 330 Cambridge Science Park, Cambridge, UK. Briefly, 12 μL of fresh 1 mM H_2O_2 was added into each well of serum samples and positive control solution and sample high control (HC) to start the reaction, then the samples were incubated at 25°C for 30 minutes, and 10 μL of stop solution added into each sample well to stop the reaction. For each well, 50 μL of developer mix containing: 46 μL of assay buffer, 2 μL of OxiRed probe, and 2 μL of horseradish peroxidase solution was prepared. Fifty microliters of the developer mix was then added to each test samples, controls, and standards. The samples were mixed well and incubated at 25°C for 30 minutes and then optical density of 570 nm in a plate reader measured [35].

Calculation: Signal change by catalase in sample was ΔA = AHC − A sample. AHC is the reading of sample HC; sample A is the reading of sample in 30 minutes. H_2O_2 standard curve is plotted, and the ΔA was applied to the H_2O_2 standard curve to get B nmol of H_2O_2 decomposed by catalase in 30 minutes reaction. Catalase activity was then calculated as follows:

$$\text{Catalase activity} = \frac{B}{30 \times V} \times \text{Sample dilution factor} = nmol/min/mL = mU/mL$$

where B is the decomposed H_2O_2 amount from H_2O_2 standard curve (in nmol); V is the pretreated sample volume added into the reaction well (in mL); 30 is the reaction time 30 minutes; one unit of catalase is the amount of catalase that decomposes 1.0 μmol of H_2O_2 per minute at pH 4.5 at 25°C.

2.2.4. Determination of MDA Concentration

Standard protocol of the Northwest Life Science Specialist, Vancouver, Canada, was used in the MDA assay. Briefly, the assay mixture in a microcentrifuge vial containing 10 μL of butylated hydroxytoluene reagent, 250 μL of serum sample, 250 μL of phosphoric acid reagent, and 250 μL of 2-thiobarbituric acid was capped and vortexed vigorously, and the mixture was incubated for 60 minutes at 60°C and then centrifuged at 10,000*g* for 2–3 minutes. The reaction mixture was transferred into a cuvette, and the absorbance of the test sample was read at 548 nm using a spectrophotometer (Spectronic-20; Philip Harris Limited, Shenstone, UK) [36].

Calculation: Plot the MDA standard curve and determine the MDA amount in the test sample in nmol by interpolation from the standard curve.

$$C = [(A/(mL))] = nmol/mL$$

where A = sample MDA amount from the standard curve (in nmol); mL = original serum volume used.

2.3. Data Analysis

Graph pad prism version 4.0 windows was used. Data obtained were expressed as mean ± standard error of mean and were subjected to Student's *t* test to determine the difference between treated and untreated horses at each period of sampling. Repeated measures analysis of variance and Tukey post hoc test were used to determine the effects of sampling periods. Values of $P < .05$ were considered significant.

3. Results

3.1. Superoxide Dismutase

The baseline values of SOD activity in the treated horses did not vary between the treated and control horses. The activity of SOD between the treated and the control horses also did not change during the first week of the experiment. On the second, third, and fourth weeks of the experiment, there was a significant ($P < .05$) difference between the treated and control horses (Fig. 1). Thus, the administration of resveratrol at these periods increased the activity of SOD in the treated horses. There was also a significant increase in the activity of SOD during the sampling periods in the treated compared with the control horses.

3.2. GPx Activity

The activity of GPx did not differ ($P > .05$) between treated and control groups on the first week of supplementation. However, GPx activity was higher ($P < .05$) in control than treated horses on the third and fourth weeks of administration of the resveratrol (Fig. 2).

3.3. Catalase Activity

The values of the catalase activity were not significantly different between the treated and control horses during periods of supplementation. However, the catalase activity increased significantly ($P < .05$) in the treated horses when the baseline value was compared with the values on the third and fourth weeks of supplementation (Fig. 3). Thus, the baseline value of the treated group was 2.60 ± 0.29

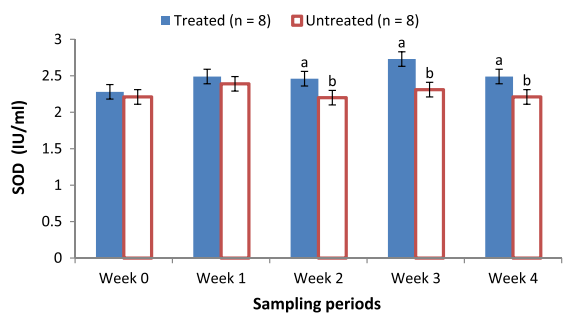

Fig. 1. Changes in superoxide dismutase (SOD) activity in horses administered with or without resveratrol. Values with different letters are significantly different ($P < .05$).

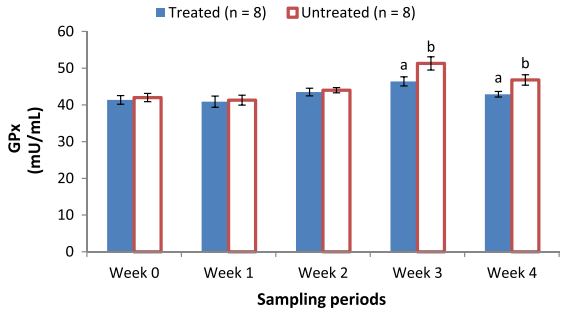

Fig. 2. Variations in glutathione peroxidase (GPx) activity in horses administered with or without resveratrol (mU/mL). Values with different letters are significantly different ($P < .05$).

mU/mL, whereas that of third and fourth weeks values were 3.5 ± 0.19 and 3.5 ± 0.28 mU/mL, respectively.

3.4. MDA Concentration

The MDA concentration did not vary between the control and treated horses during the first week of supplementation. The concentration of MDA was higher ($P < .05$) in the control than treated horses during the second and third weeks of supplementation. The highest MDA concentration (6.68 ± 1.06 μmol/mL) was measured on the second week of the experiment in the control horses. This value was higher ($P < .05$) than that recorded in the treated horses (4.04 ± 0.53 μmol/mL). There was no difference in MDA concentration of the treated and control horses on the fourth week of administration of resveratrol, although MDA concentration was lower ($P < .05$) in the treated horses than that observed on week 0, before supplementation (Fig. 4).

4. Discussion

In the present study, a significant increase in SOD activity was obtained after resveratrol supplement (Equithrive) administration. This finding may be because of stimulation of production of antioxidative enzyme, SOD, which is an important component of the vascular protective effect of resveratrol [37] present in the supplement. Previous studies have shown that resveratrol directly scavenges ROS, such as O_2, OH^-, and $ONOO^-$ [21].

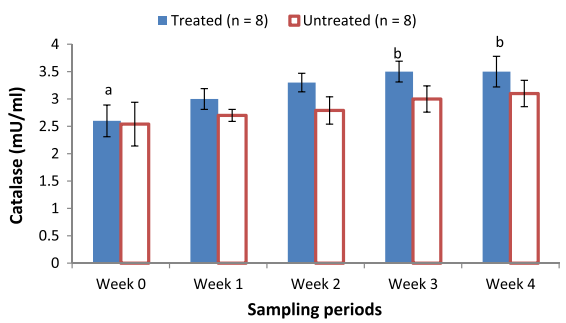

Fig. 3. Alterations in catalase activity in horses administered with or without resveratrol (mU/mL). Values with different letters are significantly different ($P < .05$).

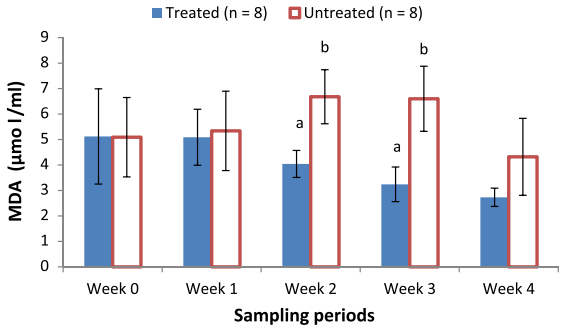

Fig. 4. Changes in malondialdehyde (MDA) concentration in horses administered with or without resveratrol (μmol/mL). Values with different letters are significantly different ($P < .05$).

Studies have shown that GPx activity increases in aged subjects [5]. On administration of resveratrol, there was a reduction in GPx activity in the horses. Work done by Villegas et al [38] on protective effect of resveratrol demonstrated that it depressed the production of GPx in the mouse spermatozoa. The result also agrees with that of Ghanim et al [39] on effects of resveratrol on oxidative and inflammatory stress in normal subjects, demonstrating a comprehensive suppressive effect on oxidative and inflammatory stress markers. Resveratrol has been found to attenuate oxidative-induced DNA damage in human lymphocytes by increasing levels of reduced glutathione and modulating activities of antioxidant enzyme, GPx, [40]. The findings of the present study disagree with those of other works in animal models, where resveratrol treatment increased the activity of GPx [41]. The difference may be because of species difference, and it requires further investigation.

The result of activity of catalase showed significant increase in the treated horses during the 4 weeks of supplementation. This reveals the capacity of resveratrol present to upregulate the activity of catalase [42,43].

The study also showed a significant reduction in MDA concentration in the treated horses on the second and third weeks of the experiment. This result agrees with the findings of previous work by Ray et al [44], Sener et al [45], and Tadolini et al [46], which reveal that resveratrol inhibits lipid peroxidation, mainly by scavenging lipid peroxyl radicals within the membrane. Indeed, the unique capacity of resveratrol to spontaneously enter the lipid environment confers on it great antioxidant potential [47]. Lipid peroxidation is a degradation process that affects structural components of the biological membranes and is among the best markers of the extent of ROS-induced biological damage [48]. Studies have examined the effects of aging on lipid peroxidation in mammalian tissues by measuring thiobarbituric acid–reactive content as a marker of endogenous lipid peroxidation [4]. Malondialdehyde, an aldehyde, is not only the end product and remnant of lipid peroxidation processes but also may act as “second cytotoxic messengers” for the primary reactions [49]. Compared with free radicals, the aldehydes are relatively stable and can diffuse within or even escape from the cell and attack targets far from the site of the original event [50].

Studies by Hichem et al [51], on ameliorative effects of resveratrol on lipopolysaccharide (LPS)-induced oxidative stress in rat liver showed that the supplement counteracted LPS-induced lipoperoxidation and depletion of SOD and catalase but slightly increased that of GPx. Some studies have evaluated the effects of resveratrol in horses [52,53]. In moderately exercised mature Quarter Horse geldings, a low (2.5 g/d trans-resveratrol) and high (5 g/d trans-resveratrol) dose had no effect on glucose tolerance, insulin sensitivity, or overall lipid peroxidation on treated compared with the control group [52]. Report by Kohnen et al [53] showed the inhibitory effect of resveratrol on equine neutrophil myeloperoxidase, whereas resveratrol treatment (1 g/d) in 20 old horses for 4 weeks resulted in decreased equine inflammatory cytokine production both in vitro and in vivo [28]. The overall findings on effects of Equithrive Joint may be due to the synergic effects of resveratrol and sodium hyaluronic acid present in the supplement. Bergin et al [54] showed an improvement in lameness conditions in horses receiving oral sodium hyaluronic acid. An unpublished study on Cortaflex (Clayton, Unpublished data, 2002), a PO administered joint nutraceutical that contains hyaluronic acid, indicated an improvement in the mobility of horses and ponies suffering from arthritic conditions of the lower limb. This reveals that coadministration of resveratrol and hyaluronic acid may help to reduce oxidative stress caused by aging and lameness in horses.

5. Conclusions

The administration of Equithrive Joint to aged and lame horses seems to decrease the serum MDA concentration and to modulate the serum content of GPx, catalase, and SOD. Such results suggest a potential protective effect of Equithrive Joint against oxidative stress and aging in horses. Further work is required to evaluate the concentration of resveratrol and hyaluronic acid in plasma of horses administered with Equithrive.

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