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Research article

Effect of hydroxytyrosol on the peroxidation of equine erythrocyte membranes by chemiluminescence

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ABSTRACT

Hydroxytyrosol (HT) is a polyphenol with a strong antioxidant effect, which is why it is being evaluated to improve animal and human health. The risk of injuries and performance of sporting horses are associated with their antioxidant status. Equine erythrocyte membranes are used as a peroxidation test model, due to their high content of double-linked fatty acids. Chemiluminescence is one of the most used methods to evaluate membrane peroxidation, due to its sensitivity and specificity. The objective of this work was to investigate the antioxidant effect of HT (POLYPHENOL - HT 1® Nova Mentis Ltd, Ireland) on the peroxidation of equine erythrocyte membranes. Erythrocyte membranes, also called ghosts, were adjusted to 1 mg of protein and challenged in a tert-butyl hydroperoxide (t-BHP)-dependent pro-oxidant model, at a 2 mM concentration and at 37 °C. Protection against peroxidation generated with HT (200 µM) was evaluated on it. Peroxidation of the ghosts was quantified in a Packard 1900 TR liquid scintillation counter using chemiluminescence in counts per minute (cpm). A completely randomized experimental design was used with the following groups: Control group (only erythrocyte membranes), 2mM t-BHP group (erythrocyte membranes + prooxidant substance) and HT group (erythrocyte membranes + prooxidant substance + HT200 µM). It was observed that the chemiluminescence value was statistically higher in the 2mM t-BHP group than in the Control, while the HT group showed an increasing reduction in chemiluminescence. The results of ten independent determinations per group show the mean and its standard deviation of the averages of 11224±2002 cpm (Control); 22000±3925 cpm (t-BHP 2mM) and 12784±2281 cpm (HT 200 µM). Data were statistically evaluated using one-way analysis of variance (ANOVA) and Tukey's test.In conclusion, HT protected equine erythrocyte membranes from peroxidation.

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Introduction

Training and competition in sporting horses generate oxidative stress, increasing the risk of injuries or loss of performance [1,2]. The evaluation of oxidative damage is frequently performed by measuring the products of membrane lipoperoxidation, especially in non-invasive samples, such as plasma or erythrocytes. In plasma, the measurement of thiobarbituric acid reactive substances (TBARS) is classic, since they are soluble compounds [3]. A study on endurance horses, subjected to 210 km of running, showed that TBARS concentrations increased with physical activity, but did so in inverse relation to the performance of the animal, being lower in the winners of the test [4]. Erythrocytes represent a non-invasive option and offer the possibility of evaluating their survival [5], their antioxidant enzymes and the oxidation state of their membrane fatty acids [6].

Different oral antioxidants have been supplemented in sporting horses to reduce oxidative stress, but their amounts must be carefully adjusted as they may have an effect opposite to that expected [7]. HT is a powerful antioxidant, available for use as a nutritional supplement, demonstrating beneficial effects in different domestic species, although it has not yet been evaluated in sporting horses [8,9,10]. A step prior to its use as an oral supplement would be to evaluate its antioxidant capacity with an in vitro test on erythrocytes from horses in training. The antioxidant capacity can be evaluated on whole erythrocytes or on their membranes (ghost). A test on whole erythrocytes, taken from healthy humans, demonstrated that the addition of HT inhibited the peroxidation of their membrane lipids in direct relation to their concentration in the medium. However, when whole erythrocytes are used, intracellular antioxidant enzymes must be taken into account, such as glutathione reductase [4], catalase and superoxide dismutase [11], which may vary with HT supplementation. in the middle. The use of erythrocyte membranes allows the assessment of damage levels in a more limited way, without so much interference from defense mechanisms, and the degree of peroxidation of their lipids is a

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sensitive tool of oxidative damage [12]. In this sense, an in vitro study with erythrocyte membranes taken from humans with different diseases (obesity, diabetes and hypertension) demonstrated that the addition of HT in the media reduced lipid peroxidation [13]. These authors evaluated TBARS levels. Another option to evaluate the degree of lipid peroxidation is chemiluminescence, characterized by providing good sensitivity and specificity [14].

Previous studies by our group estimated that the ideal concentration of HT (Chemical structure of Hydroxytyrosol is given in Fig. 1) to protect mitochondria from membrane peroxidation was 200 µM [15]. Considering that this concentration could protect erythrocyte membranes from horses in training, its effect on the peroxidation of these membranes was evaluated by chemiluminescence.

Materials and Methods

Animals

Ten blood samples were obtained from ten adult horses, weighing between 450 and 550 kg, obtained from the Teaching Hospital of the Faculty of Veterinary Sciences of the National University of La Plata.

Materials

BSA (fraction V) was obtained from Wako Pure Chemical Industries Ltd, Japan. L (+) ascorbic acid, dimethyl sulfoxide and methanol were from Merck Laboratories and POLYPHENOL - HT 1® was obtained from Nova Mentis Ltd, Ireland. All other reagents and chemicals were of analytical grade from Sigma-Aldrich.

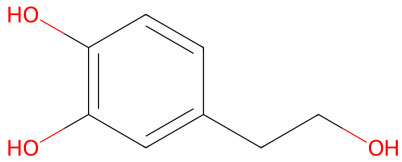

Figure 1: Chemical structure of Hydroxytyrosol.

Preparation of erythrocytes

Blood was obtained by jugular venipuncture using EDTA as an anticoagulant. The erythrocytes were isolated from whole blood by centrifugation (1000g for 10 min at 4 °C). The buffy coat and plasma were discarded and erythrocytes were washed three times in isotonic phosphate buffer (PBS 5 mM pH 7.4, 150 mM NaCl). The erythrocytes pellet was suspended in isotonic phosphate buffer.

Preparation of erythrocyte ghosts

Preparation of erythrocyte membranes (ghosts) was carried out according to the method of James T. Dodge with modifications. Briefly, packed, washed erythrocytes were lysed by adding 10 vol of 5 mM phosphate buffer pH 7.4 (at 4 °C) while mixing [16]. After leaving on ice for 30 min, the erythrocyte membranes were packed by centrifugation at 20,000g for 10 min at 4 °C and the hemoglobin-containing supernatant was removed. The erythrocyte membranes were then washed three times by suspending in fresh buffer (same volume as used for lysis) followed by centrifugation under the same conditions. Finally, the membranes were suspended in isotonic 5 mM buffer (same volume as used for lysis) followed by centrifugation under the same conditions and then resuspended in isotonic 5 mM phosphate buffer pH 7.4. Erythrocyte membranes were quantified on the basis of protein concentration using the assay of Oliver H. Lowry [17].

Erythrocyte membranes peroxidation

Erythrocyte membranes, were adjusted to 1 mg of protein and challenged in a tert-butyl hydroperoxide (t-BHP)-dependent pro-oxidant model, at a 2 mM concentration and at 37 °C.

The erythrocyte membranes were incubated with HT 200 µM in an in vitro non-enzymatic ascorbic acid-Fe^{+2} system in order to determine the oxidative effect on membranes and quantify peroxidation level in standarized conditions.

Peroxidation was quantified in a liquid scintillation counter Packard 1900 TR by chemiluminescence in cpm (counts per minute). Erythrocyte membranes without HT were used as a control. Chemiluminescence and peroxidation were initiated by adding t-BHP. The erythrocyte membranes with addition of HT 200 µM were incubated at 37 °C. Membrane light emission was determined over a 50 min period, chemiluminescence was recorded as count per minute (cpm) every 10 min and the sum of the total chemiluminescence was used to calculate cpm/mg protein. Chemiluminescence was measured as counts per min in liquid scintillation analyzer Packard 1900 TR equipment (Japan) with a program for chemiluminescence.

Protein determination

Proteins were determined by the method of Oliver H. Lowry using BSA as standard [17].

Statistical analysis

Results are expressed as means ± S.D. of ten independent determinations. Data were statistically evaluated by one-way analysis of variance (ANOVA) and Tukey's test. The statistical criterion for significance was selected at different *p*-values, which was indicated in each case.

Results

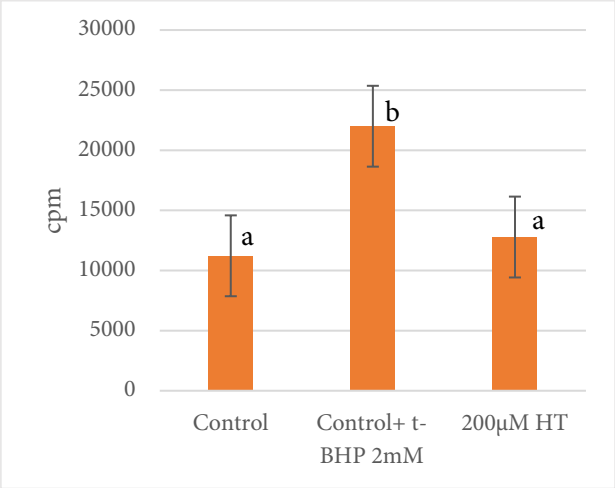

Figure 2: Total light emission obtained from the HT group and the control group.

Incubation of the equine erythrocyte ghosts in the presence of t-BHP resulted in peroxidation of the membranes, as evidenced by light emission (chemiluminescence). After incubation of the ghosts with t-BHP at 37 °C for 180 minutes (cpm every 10 min), the cpm caused by light emission were lower in the HT group than in the control group.

The Figure 2 shows the total light emission obtained from the HT group and the control group. When comparing the control group with the group in the presence of HT, it was observed that in the group treated with HT there was protection of the membranes. Count per minute (mean ± SDM) was 11224

± 2002 cpm in the control; in control + t-BHP 22000±3925 cpm and control + t-BHP 2mM + HT 200µM 12784±2281 cpm

Results are expressed as mean ± SD of ten independent experiments. The values of light emission showed statistically differences ($p<0.05$), when the control and control + 2mM t-BHP groups were compared. Similarly, differences ($p<0.05$) were found when was compared with t-BHP and HT 200µM antioxidant addition.

Discussion

Training increases oxidative stress in horses, especially during post-training tissue repercussion, increasing the levels of lipoperoxidation, expressed in malondialdehyde (MDA) concentrations in plasma [18]. Increasing levels of MDA are also associated with apoptosis in muscle cells [19]. Erythrocytes are a valuable option in sporting horses to evaluate oxidative damage from a much less invasive sample than biopsies [20]. However, the whole erythrocyte can respond to oxidative stress with changes in the activity of antioxidant enzymes, such as SOD, changing the response to a pro-oxidant [11]. The evaluation of membranes (ghost) is ideal to measure lipoperoxidation and the protective effect of antioxidants on [13].

HT being a powerful antioxidant, its effect has been evaluated associated with physical training in laboratory animals, but not yet in horses. The antioxidant effect of HT is not only based on the protection of sensitive structures, such as membranes, but also with improvements in mitochondrial function [21] and increases in the expression of genes associated with fatty acid oxidation., lactate and glucose [22]. In rats, the antioxidant capacity of HT is directly associated with its concentration in the diet in sedentary animals, but in trained animals, a HT intake of 20 mg/kg is antioxidant, but with 300 mg/kg it has a protective effect oxidant [23].

The concentrations of HT that have been shown to be most efficient to protect from oxidative damage in vitro have been 50 µM with rat liver microsomes and 200 µM with rat liver mitochondria, using ascorbate-Fe2+ as a pro-oxidant [15,24]. In the present work, t-BHP was used to attack membranes of horses undergoing training, and the 200 µM concentration was equally effective in generating the protective effect of HT.

Conclusions

With this background, different doses of HT as an oral supplement should be evaluated in horses in training evaluating peroxidation in the erythrocyte membranes as described in this work. In this study, it was noticed that the chemiluminescence value was statistically higher in the 2mM t-BHP group than in the control, while the HT group showed an increasing reduction in chemiluminescence. The results of ten independent determinations per group show the mean and its standard deviation of the averages of 11224±2002 cpm (Control); 22000±3925 cpm (t-BHP 2mM) and 12784±2281 cpm (HT 200 µM). Data were statistically evaluated using one-way analysis of variance (ANOVA) and Tukey's test. The HT succeeded in protecting equine erythrocyte ghosts from peroxidation was observed.

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