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Abstract

The present investigation was carried out to determine the presence of oxidative alterations in the horses erythrocyte membrane during a high intensity exercise test. The degree of peroxidation was estimated by chemiluminescence using a suspension of lysed erythrocytes incubated with t-butyl hydroperoxide (t-BHP). Differences were observed in the total values of chemiluminescence throughout the exercise routine, with higher values of light emission obtained with the animal at rest in relation to those observed during and after exercise.The conclusions of this study are the existence of changes in the erythrocyte membranes of the horses exposed to physical exertion, probably associated with the release of ROS caused by the exercise and that the determination of chemiluminescence in suspension of lysates erythrocyte is a sensitive assay applied to detect the existence of oxidative stress associated to physical exercise.

Index terms— oxidative stress; exercise; chemilumine-scence; erythrocyte; tert-butyl hydroperoxide

uring the exercise, there are several potential sources to produce reactive oxygen species, which can produce oxidative stress. Exercise generates different types of physiological responses in an individual that depend on the type and duration of the same, since it supposes a stress for the organism that tests its capacity of adaptation (Art and Lekeux2005; Vollaard et al. 2005; Posada Arias et al. 2013). During exercise, oxygen consumption (VO 2) is increased, which is used to produce energy in the mitochondria of muscle fibers, generating intermediate species called reactive oxygen species (ROS) **??**Inayama et al. 2000; **??**ernandez et al. 2009). In blood, the oxidation of oxyhemoglobin to methaemoglobin generates a large amount of ROS, the value of which is directly related to the type of exercise performed and the need for oxygen in the tissues (Clemens and Waller 1987;Svistunenko 2005). The ROS production during exercise depends on the intensity, frequency, duration and type of exercise (Williams et al. 2005; Kirschvink et al. 2008). Therefore, the exercise is considered as a condition of excessive generation of ROS, which also results in compensatory compensations by the antioxidant systems (Vollaard et al. 2005), however, ROS generation can become overwhelming for the antioxidant defense system and pose potential problems, inducing the loss of membrane integrity and cellular dysfunctions, affecting cellular lipids, proteins and DNA (Clarkson and Thompson 2000). In relation to blood cells, circulating erythrocytes are regularly exposed to stress conditions and are especially vulnerable as they have no membrane repair mechanism or regenerative capacity. Due to the high tension of O 2 in arterial blood and the content of Fe, within erythrocyte continuously occur ROS such as O 2 (-), H 2 O 2 and HO (Bakker et al. 2000;Cimen 2008;Herlax et al. 2011).

It is known that ROS readily attack polyunsaturated fatty acids (PUFAs), present in cell membranes, such as the erythrocyte, a process known as lipid peroxidation (oxidative destruction of PUFAs) (Dillard et al. 1978). Oxidative lipid damage can lead to disorganization, dysfunction and destruction of membranes (Halliwell and Gutteridge 1990). This may be due to a decrease in their fluidity, inactivation of receptors and enzymes, increased ion permeability and eventually membrane rupture (Gutteridge and Halliwell 1990;Gutteridge 1995). The presence of oxidative stress does not automatically imply oxidative damage. Oxidative stress has been defined as the exposure of cells to various sources that produce a break in the balance between the pro-oxidant factors and the antioxidant mechanisms responsible for eliminating these chemical species, either by a deficit of these defenses or by an exaggerated increase of the production of ROS. All this results in alterations of the structure-function relationship in any specialized organ, system or cell group (Venereo Gutierrez 2002). Oxidative damage can only be verified by direct measurement of different markers of this process. Peroxidation is the biomarker of

oxidative damage most extensively studied after exercise (Deaton and Marlin 2003). Various studies in human and veterinary medicine have been developed for the analysis of peroxidation in red blood cells, with the exposure to a large number of prooxidants agents such as: cumenehydroperoxide

1 a) Materials

The tert-BHP was obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents and chemicals were of analytical grade.

2 b) Animals

Eight adult horses, weighing between 450 and 470 kg and belonging to University farm, were used in the assay. Horses were maintained on alfalfa bale and tap water ad libitum.

The horses were accustomed to continuous training on a treadmill (Kagra, Mustang 2200) which is in the Laboratory of Physiology and Pathophysiology of Equine Sport, Faculty of Veterinary Sciences, National University of La Plata.The animals were given the following standardized exercise protocol: preheating 1 min at 1.5 m/s and 4 min at 4 m/s; then, with a 3% slope, 1 minute steps were performed with increasing intensities (5; 6; 7; 8; 9; 10; 11; 12; 13 m/sec, etc.) until reaching the fatigue point. Finally, the recovery phase was performed without slope at 4 and 1.5 m/s for 4 and 1 min respectively (Muriel 2016).Peripheral blood samples were obtained from the right jugular vein (previous channeling) in heparinized tubes. Samples were taken with the animal at rest prior to exercise (T0 or rest), at the fatigue point (T1 or exercise) and at the end of recovery (T2 or recovery) (Muriel 2016).All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

3 c) Preparation of erythrocytes

Samples were quantified based on hemoglobin concentration, determined by photometry on a Sysmex KX21-N hematology analyzer (Sysmexcorporation, Kobe, Japan). 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 mMNaCl). The erythrocytes pellet was suspended in isotonic phosphate buffer. Preparation of suspension of lysates erythrocyte was carried out according to the method of Dodge et al. (1963). Briefly, packed, washed erythrocytes were lysed by adding 10 vol of 5 mM phosphate buffer pH 7.4 (at 4°C) while mixing and after leaving on ice for 30 min. Finally homogenizing the suspension.

4 d) Peroxidation of erythrocyte analyzed by chemiluminescence

Suspensions of lysates erythrocyte were incubated at a final concentration of 0.25 mg/ml total hemoglobin with 2 mM t-BHP for 40 min at 37°C. Identical aliquots of the preparation were incubated for 40 min at 37°C without addition of t-BHP as the control experiment for endogenous peroxidation products in the erythrocyte lysates preparation.

Peroxidation was initiated by adding a small amount of stock solution of t-BHP (80 mM) to each vial that was maintained at 37°C and was measured by monitoring light emission (Wright et al. 1979) with a liquid scintillation analyzer Packard 1900 TR. Chemiluminescence was determined over a 40 min period and recorded as count per minute (cpm) every 10 min.

5 e) Statistical analysis

Analysis of variance and student's t-test.was performed to test the significance of difference ($P<0.05$) between the mean values among groups.

6 f) Results

The addition of t-BHP to equine suspension of lysates erythrocyte resulted in the peroxidation as evidenced by the emission of light. All results are shown in Table 1. Differences were observed in the total values of chemiluminescence throughout the exercise routine, with observed values of 331.620cpm (± 26.324), 243.290cpm (± 15.875) and 242.630cpm(± 8.351) for T0, T1 and T2 respectively. The values obtained were different between T0 and T1 and between T0 and T2 ($p = 0.0413$ and 0.0131 respectively). There were no differences between T1 and T2.

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Figure **??** shows the total chemiluminescence during incubation of equine suspension of lysates erythrocyte with or without the addition of t-BHP.

The higher value of chemiluminescence reached with addition of t-BHP was a 425.002 cpm (equine 7, TO) while the minimum value was 174.860cpm(equine 5, T1).The data are given in Fig. **??**. It is known that horses

are exposed to exerciseinduced changes in oxidative/antioxidant balance, depending on the type of exercise, intensity and duration, training level, environmental conditions, and the presence of diseases (Williams et al. 2005(Williams et al. , 2012)). In this specie, the occurrence of oxidative stress induced by exercise has been well demonstrated (Hargreaves et al. 2002;Kirschvink et al. 2002). Both training and exercise induce the production of ROS which cause cell and tissue damage (Clarkson and Thompson 2000).The mechanics of ROS generation are not completely clear, although its sources include the oxidation of hemoglobin in the same blood and the processes of ischemia-reperfusion in various tissues (Van der Zee 1996; Domanski et al. 2004;Svistunenko 2005;Muriel 2016). These mechanisms may act synergistically and their magnitude is related to the type of exercise performed and its intensity (Finaud et al. 2006).Respect to the ischemia-reperfusion mechanism, during exercise the flow of blood is restricted in some areas (kidneys and splanchnic region) to be diverted to the active muscles. This produces a hypoxia state in restricted areas, directly related to the magnitude of the exercise (Adams and Best 2002). Also, muscles undergo relative hypoxia during exercise performed at intensities above maximal oxygen consumption, since the supply cannot meet the energy needs (Powers and Jackson 2008). Finally, reoxygenation of these tissues, known as payment of oxygen debt, occurs after cessation of exercise, which leads to an increase in ROS generation (Ji 1999).

In the present study, suspension of lysates erythrocyte from equine submitted to a high intensity exercise, were exposed to a prooxidant (t-BHP). Erythrocytes have many scavenger systems, and can be used to examine the balance between pro-oxidants and antioxidants since they are representative cells where superoxide radicals are being continuously generated by auto oxidation of hemoglobin. We used lysed red cells because we believe it is a relatively simple model, since in these cells the presence of redox-active hemoglobin residues, with peroxidative activity, potentially catalyzes the oxidation of membrane components including polyunsaturated lipids (Everse et al. 1994 Lipid peroxidation is by far the most extensively studied marker of oxidative damage following exercise (Deaton and Marlin 2003). Although it is possible to have chemiluminescence without lipid peroxidation in cell-free systems, it is established that an increase in lipid peroxidation rate in organs and isolated cells produces a parallel increase in photoemission. We observed the existence of changes in the erythrocyte membranes of the horses subjected to physical exertion, these findings clearly suggest the prooxidant environment prevailing in the blood during highintensity exercise, probably associated with the release of ROS caused by the exercise. [1]

estimating the degree of peroxidation by chemiluminescence.

[Note: (Akoev et al. 1998;Tesoriere et al. 2001), t-butyl hydroperoxide (t-BHP)(Mawatari and]

Figure 1:

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[Note: Kabc : means with different superscripts differ significantly at p<0.05]

Figure 2: Table 1 :

Figure 3:

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