

# Effect of Exercise on Hematological Values and its Relationship with Erythrocyte Membrane Damage in Horses



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## Abstract

Physical exercise is considered a stress for the animal organism because it generates different types of responses, as are the negative effects caused by free radicals. In this paper, the hematologic response level of red blood cells and the presence of oxidative alterations in their membranes, in horses subjected to a test of high-intensity exercise were evaluated. The results indicate a significant increase in the number of red blood cells, hematocrit and hemoglobin concentration, reaching maximum values when the horses reach fatigue during exercise test, descending during aerobic recovery. The three parameters behaved similarly. In relation to damage the membrane of red blood cells, it was estimated by the degree of peroxidation, measured by chemiluminescence, over the exercise period. The values produced by peroxidative damage are high at the time of exercise exhaustion, remaining stable after recovery. The correlation between the studied hematological values and the degree of damage of the erythrocyte membrane shows that, despite of the increased circulating red blood cells because of splenic contraction, the erythrocytes are damaged due to the predominant oxidizing environment during the maximal exercise

**Keywords:** Horse; Red blood cells; Exercise; Chemiluminescence; Hematocrit

## Introduction

Exercise generates different responses in an individual depending on the type and duration, being a stress situation to the organism that tests its adaptability [1]. Regarding the hematological system, several authors (both in humans and different animal species research) describe changes in blood volume, population of white and red cells and in the count and shape of platelets, considering such changes being related to exercise [2-6]. It has been determined that the increase in the number of erythrocytes during exercise is mainly due to a process of splenic contraction by sympathetic stimulation [6], as a result of processes of hypoxia and acidosis generated during intense exercise, which in turn leads to a stronger splenic contraction [7]. Studies in different animal species show, when considering erythrocytes and after a vigorous and prolonged exercise, increases in total cell mass within a proportionally greater plasma volume [8-13]. Horses can rapidly increase the oxygen carrying capacity of blood by increasing hemoglobin concentration through splenic contraction [10,14]. It can be stated that, when considering parameters related to erythrocytes (cell count, hematocrit and hemoglobin concentration), values are often increased in the early stages of an exercise (splenic contraction). After long-term exer

cise, the expansion of blood volume and the subsequent dilution effect (body fluids redistribution), cause a decrease in the relative values of these parameters, although they vary according to the degree of dehydration caused by the exercise itself [15].

In aerobic organisms, oxygen (O<sub>2</sub>) is essential for life as it is fundamental for energy metabolism, but it can also be toxic and is implicated in numerous diseases and degenerative conditions [16]. Oxygen is used as the terminal oxidant in the mitochondrial respiratory chain, however, the presence of intracellular O<sub>2</sub> can result in the occurrence of redox reactions which may damage biomolecules [17]. Formation of free radicals during exercise depends on the intensity, frequency, duration and type of it. High levels of oxygen consumption during exercise have also been implicated as a contributing factor to oxidative stress [18-21]. Circulating erythrocytes are regularly exposed to stress conditions and are particularly vulnerable because they have no mechanisms of membrane repair or regenerative capacity [22]. During exercise, the oxidation of oxyhemoglobin to methemoglobin generates a great number of free radicals by release of O<sub>2</sub><sup>-</sup>, a condition that is directly related to the type of exercise and the oxygen need for in tissues [22]. Oxidative damage can only be verified by

direct measurement of different markers of this process. Peroxidation is by far the biomarker of oxidative damage most extensively studied after exercise [23]. Several assays in both human and veterinary medicine have been developed to study peroxidation in red blood cells, such as the addition of various pro-oxidants like cumene hydroperoxide [24] tert-butyl hydroperoxide (t-BHP) [25-27] and fatty acid hydroperoxides [13,28] which have been made from suspensions of red cell ghosts [13,14,25-28] and also from lysed cells [29-32]. Aims of this study were to analyze de oxidative alterations in the erythrocyte membrane of horses submitted to a high intensity exercise test by estimating the degree of peroxidation by chemiluminescence and to evaluate the relationship between these changes and the variations in the hematological values during different sates of exercise.

## Materials and Methods

### Experimental animals

Five adult horses, 3 purebred Arabians and 2 half-Arabians, were used. Animals were in excellent nutritional and health status and had an average weight of 440kg. They were housed in boxes at the facilities of the Hospital School of the Faculty of Veterinary Sciences, National University of La Plata, all throughout the test period. Animals were fed based of body weight (2.5% dry matter). Diet exhibited a 50:50 ratio of hay (roll pasture) and concentrated (balanced feed with 12% protein and 2.75Mcal/kg); water was administered ad-libitum.

### Standardized exercise test

Standardized exercise tests are tests of physical exertion with controlled speed, gradual in many cases, which allow to reproduce or simulate a maximum effort situation under controlled conditions. Animals performed the test at the Laboratory of Physiology and Pathophysiology of Sport Horse, Faculty of Veterinary Sciences, National University of La Plata. For this purpose, a horse treadmill (Kagra, model Mustang 2200) was used, located in a building with the following dimensions: length 14.6m, width 9 meters and 10 meters high. The facility has a non-slip rubber floor to prevent horses from slipping, two frontal fans to simulate wind drag when the animal runs on a track, and a wall clock that records barometric pressure (mbar), temperature (°C) and humidity (%), to know the environmental conditions where the animal performs physical effort.

Technical characteristics of the treadmill are:

- A. Length: 10.3 meters.
- B. Width: 3.64 meters.
- C. Floor to band height: 0.55 meters.
- D. Maximum height: 4.3 meters.
- E. Weight: 3770 kg.
- F. Total dimensions: 4 meters long and 1.2 meters wide.

- G. Machine speed: range 0-15 m/sec.
- H. Incline percentage: range 0 to 11%.
- I. Permanent load factor: up to 700 kg.

### Exercise protocol

Prior to the start of the exercise tests all the horses had an adaptation period to the treadmill consisting of ascending and descending, walking, trotting and galloping. Standardized exercise test consisted of 1min preheating at a speed of 1.5m/sec. and then 4min at 4m/sec, followed by a 3% slope steps of 1min with increasing intensities (5; 6; 7; 8; 9; 10; 11; 12; 13 m/sec, etc.) until fatigue. Then, the recovery phase was as follow: 4 and 1.5m/s without slope during 4 and 1min, respectively [14]. Three tests were performed on each animal with at intervals not less than one week.

### Blood sampling

For blood collection during tests, an antiseptic was applied to the skin and jugular catheterization was performed with a #14 Abbocath which was fixed with a suture to the skin. Then the catheter was connected to a flexible tube (both heparinized) long enough to gather blood sample without interrupting the exercise.

Blood samples were taken:

- A. Before exercise (T0 or rest)
- B. At fatigue (T1 or exercise)
- C. After the recovery phase (T2 or recovery) [14].

Samples (5ml blood) were placed in heparinized tubes, immediately homogenized and processed at the end of the extraction of the three samples.

### Sample processing

Hematology: First processing stage consisted of hemogram determinations (RBC count, hematocrit and measurement of hemoglobin concentration). Tests were performed at the Central Laboratory, Faculty of Veterinary Science, National University of La Plata, using a Sysmex KX 21 automated hematology analyzer.

### Preparation of erythrocytes

Red cells were isolated from whole blood by centrifugation (1000g for 10min at 4°C). The buffy coat and plasma were discarded, and erythrocytes were washed three times in isotonic phosphate buffer (PBS 5mM pH 7.4, 150mM NaCl). The erythrocyte pellet was suspended in isotonic phosphate buffer. Preparation of suspension of erythrocyte lysates was carried out according to the method of Dodge et al. [33]. Briefly, packed, washed erythrocytes were lysed by adding 10vol of 5mM phosphate buffer pH 7.4 (at 4°C) while mixing and after leaving on ice for 30min. Finally, the suspension was homogenized.

**Peroxidation of erythrocyte analyzed by chemiluminescence**

Peroxidation of erythrocyte lysates with final concentration of 0.25mg/ml total hemoglobin was initiated by adding of t-BHP (80mM) to each vial (T0, T1 and T2). Erythrocyte lysates preparations, which lacked t-BHP, were carried out as control simultaneously. Membrane light emission was determined over a 40min period at 37°C, chemiluminescence was recorded as count per minute (cpm) every 10min and the sum of the total chemiluminescence was used to calculate total cpm. Chemiluminescence was measured as counts per min in liquid scintillation analyzer Packard 1900 TR equipment with a program for chemiluminescence [34].

**Statistical Analysis**

Data obtained were considered independently for each of the determinations and are presented as the means ± SE. Test Student's t was performed, assuming a single distribution and equal variances between samples to analyze the presence or absence of significant changes (P<0,05) between the means of the different experimental values (T0, T1 and T2). Possible associations between variables analyzed (hematological values and peroxidation) were performed through Pearson correlation coefficient (SPSS® Version 11).

**Results**

**Hematologic values**

Table 1 and Figure 1 show the parameters of the red series evaluated for this study. Table 1 shows the individual results of the three blood parameters analyzed for each of the samples obtained during the exercise test. In T0 the average number of erythrocytes was 8,44x10<sup>6</sup> cell/μl (±0,32), 11,65x10<sup>6</sup> cells/μl (±0,25) in T1 and 10,15x10<sup>6</sup> cells/μl (±0,26) in T2 (Figure 1). Hematocrit increased during the initial stage of exercise test, with a baseline of 35,4% (±0,9) at T0 that increased to 53,6% (±0,4) in T1, and finally descended to 45,9% (±0,9) in T2 (Figure 1). Regarding hemoglobin concentration, parameter showed a similar trend, ranging from 12,93 g/dl (±0,2) to 18,09 g/dl (±0,2) and 15,63 g/dl (±0,3) for T0, T1 and T2, respectively, (Figure 1). Although the three parameters presented a similar distribution throughout the exercise routine, the initial values (T0) were only different from those observed in T1 for hematocrit (p=0,021), and for hemoglobin concentration (p=0,010); the differences observed for the total number of red blood cells are not statistically significant. No significant differences were found when analyzing the values obtained after finalization of the test period (T2), in relation to the initial values (T0) and data from the samples obtained at the time of fatigue (T1).

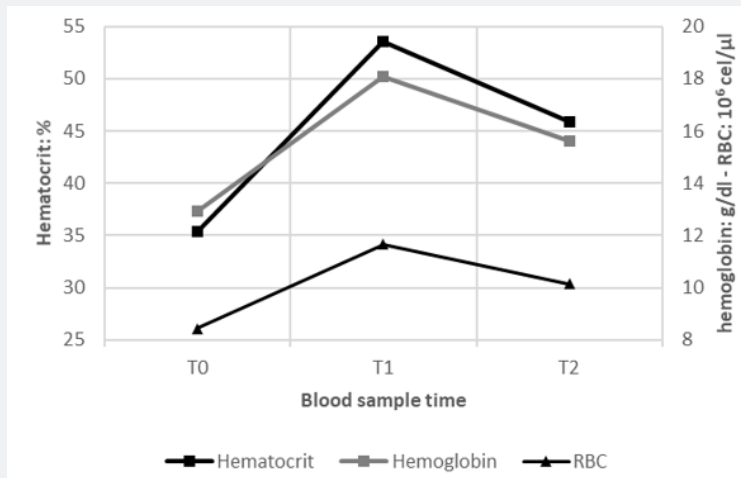


Figure 1: Hematologic values.

Table 1: Hematologic values.

Parameter	Extraction time	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Means ± SE
Hematocrit %	T0	40,1	38,6	38,0	33,2	36,8	34,2	36,8	31,9	31,5	35,3	38,1	37,2	26,4	35,8	36,5	35,4±3,5 <sup>a</sup>
	T1	55,6	52,4	53,0	51,0	52,7	54,3	55,9	52,9	52,1	55,3	52,3	53,4	55,6	55,1	52,0	53,6±1,6 <sup>b</sup>
	T2	50,2	42,6	45,0	44,7	41,5	49,2	50,2	46,4	48,3	44,8	42,0	41,9	51,6	46,5	43,8	45,9±3,4 <sup>b</sup>
RBC 10 <sup>6</sup> cel/μl	T0	10,0	10,1	9,5	7,3	7,7	7,2	8,4	7,0	6,7	7,4	10,3	9,7	9,2	8,5	7,7	8,4±1,2
	T1	13,6	12,5	13,2	10,7	10,6	10,9	12,2	10,9	10,7	11,2	12,6	11,3	12,0	11,3	11,1	11,6±1,0
	T2	12,4	10,6	11,2	9,5	8,6	9,9	11,0	9,7	9,8	9,3	11,1	9,5	10,9	9,6	9,4	10,2±1,0

He- mo- globin g/dl	T0	13,3	12,9	13,2	12,4	13,5	12,5	13,2	11,8	11,9	13,1	12,8	13,6	13,5	13,8	12,5	12,9±0,6*
	T1	18,3	16,4	17,1	17,7	18,6	19,1	18,9	18,1	18,7	19,7	16,8	17,2	18,6	18,5	17,6	18,1±1,9#
	T2	16,0	14,2	13,7	15,7	14,9	17,3	17,0	16,2	16,3	16,1	13,2	14,5	16,7	16,8	15,9	15,6±1,2#

ab; #: means with different superscripts differ significantly at p<0,05.

**Degree of peroxidation**

The addition of t-BHP to the suspension of erythrocyte lysates resulted in their peroxidation, as evidenced by the light emission (Table 2). When comparing the values obtained at the three times (T0, T1 and T2), with or without the addition of t-BHP, these were always statistically different (p<0,001). The

total chemiluminescence values (total cpm) in samples with the addition of t-BHP were 342.830cpm (±21.013), 242.258cpm (±11.367) and 236.256cpm (±8.245) for T0, T1 and T2 respectively. These results are shown in Figure 2. The values were significantly different between T0 and T1 (p=0,011) and between T0 and T2 (p=0,003). There were no differences between T1 and T2.

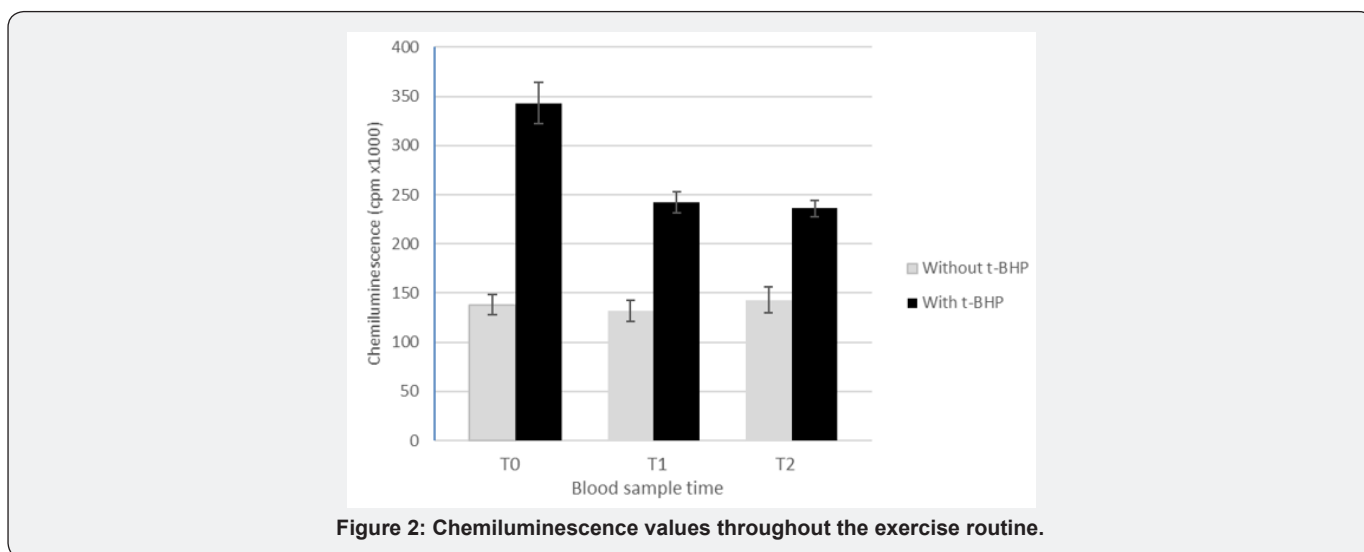


Figure 2: Chemiluminescence values throughout the exercise routine.

Table 2: Time course of light emission (cpm x 1000).

Ex-traction time	Treat-ment	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Means± SE
T0	With t-BHP	260,1	370,9	281,7	271,2	401,2	246,1	400,5	425,0	277,9	495,5	401,2	252,5	401,2	401,2	256,2	342,8±10 <sup>a</sup>
	With-out t-BHP	90,5	139,2	181,3	174,9	149,4	89,6	115,2	187,3	91,9	180,0	145,1	92,7	114,9	187,3	127,9	137,8±11 <sup>b</sup>
T1	With t-BHP	250,4	262,9	193,0	184,2	256,2	174,9	250,7	315,5	251,5	308,7	271,2	251,5	174,9	262,9	225,5	242,3±11 <sup>c</sup>
	With-out t-BHP	98,2	99,5	112,0	122,9	150,2	93,9	78,1	145,1	190,7	199,2	150,2	94,9	89,6	174,9	185,9	132,3±21 <sup>b</sup>
T2	With t-BHP	234,9	275,4	141,3	210,2	243,2	224,2	234,3	278,7	240,3	231,0	250,7	234,7	234,3	260,1	250,7	236,3±11 <sup>c</sup>
	With-out t-BHP	108,0	185,9	127,9	114,9	156,3	103,3	93,9	234,7	200,9	95,9	99,5	105,3	193,0	210,2	114,9	143,0±8 <sup>b</sup>

abc: means with different superscripts differ significantly at p<0,05.

**Correlation between variables**

A high positive correlation was found by comparison of the three hematologic variables analyzed (hematocrit, red blood cell count and hemoglobin level), regardless the time of sam-

ple collection. Conversely, individual comparison of these variables with the values of chemiluminescence after the addition of t-BHP, resulted negative. These results show that, despite the increment caused by the exercise test in the number of red blood

cells and their associated variables (hematocrit and hemoglobin), membranes of these cells demonstrated a greater damage, as evidenced by the lower degree of chemiluminescence.

## Discussion

The organism responds to physical demands, such response is related to the type and duration of the activity. This response is manifested in several organic systems and the hematological system is not the exception, in particular the red blood cells studied in this work. The literature mentions that hemoconcentration takes place at the beginning, being this attributable to dehydration as a consequence of fluid loss through perspiration. Also, an increase in hemoglobin concentration, hematocrit and number of erythrocytes while plasma volume decreases, as well as total blood volume. When physical challenge is more demanding or occurs over a longer time, these variables tend to increase [6]. The increments in hemoglobin, hematocrit and red blood cells observed in this work, leads to hemoconcentration, determined by the mobilization of erythrocytes from the spleen [35] and the fluid output to the extravascular space [36]. The increments in these variables were similar.

In horses during rest, about 33% of the erythrocytes are stored in spleen. In the present study increments were observed for the three hematological variables analyzed when horses reached fatigue (T1); values decrease after recovery but remain higher compared to T0. In relation to the damage of red blood cell membranes, there is increasing evidence of changes induced by exercise in the oxidant / antioxidant balance, depending on the type, intensity and duration of exercise [20]. This is due to the production of reactive oxygen species, which cause cell and tissue damage [37]. Many studies have documented the oxidative stress induced by exercise by measuring oxidative damage to cellular components. Among the biomarkers of oxidative damage, peroxidation is a widely used method [23]. In this study, peroxidation of erythrocyte membranes was evaluated by measuring the light emission in suspensions of lysed erythrocytes of horses subjected to high-intensity exercise, exposed to an oxidant (t-BHP). It is established that an increase in the peroxidation rate produces a parallel increase in the photoemission. Since oxygen radicals are produced continuously in erythrocytes by autoxidation of hemoglobin and that erythrocytes have mechanisms for protection against oxidative damage, including catalase [38] superoxide dismutase [39] and low molecular weight antioxidants such as ascorbate [40] we must consider that the existence of changes in erythrocyte membranes, evaluated by peroxidation, is a consequence of physical exercise. Results clearly suggest the pro-oxidizing environment prevailing in blood during high-intensity exercise, probably associated with the release of ROS induced by such circumstance [41-43].

When relating the tested hematological parameters (number of RBC, hematocrit and hemoglobin) with the chemiluminescence values obtained in the three periods analyzed, an inverse

correlation is observed. The latter may be attributable to the fact that recirculating erythrocytes show considerable damage in their membranes, due to the oxidative environment prevailing in the stage of maximum exercise, which is maintained during the recovery stage, although there is an increase in hematological values mainly due to splenic contraction.

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## Compliance with ethical standards

Procedures involving animals were in accordance with the recommendation of the Bioethics Committee of UNLP (National University of La Plata).

## Conflict of Interest

All authors have no conflict of interest in this study.

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