Effect of exercise on hematological values and its relationship with erythrocyte membrane damage in horses

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ABSTRACT

Physical exercise is considered as stress for animal organism because it generates different types of responses, as negative effects caused by free radicals. In this study, the hematologic response level of red blood cells and the presence of oxidative alterations in the membranes of horses subjected to a test of high-intensity exercise were evaluated. The results indicated 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, and then decreased during aerobic recovery. The three parameters behaved similarly. Damage to the membrane of red blood cells 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, and remained stable after recovery. The correlation between the studied hematological values and the degree of damage of the erythrocyte membrane showed that, despite the increased circulating red blood cells due to splenic contraction, the erythrocytes are damaged as a result of the predominant oxidizing environment during the maximal exercise.

Key words: 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 (Posada Arias et al., 2013). Regarding the hematological system, several authors (both in humans and different animal species research) describe changes in blood volume, population of white and red blood cells and in the count and shape of platelets, considering such changes being related to exercise (Novosadova, 1970; Myhre et al, 1985; Schmidt et al., 1989; Remacha et al., 1993, Bonilla, 2005). 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 (Bonilla, 2005), as a result of processes of hypoxia and acidosis generated during intense exercise, which in turn leads to a stronger splenic contraction (Leleu et al., 2005). Studies on different animal species showed, when considering erythrocytes and after a vigorous and prolonged exercise, increases in total cell mass within a proportionally greater plasma volume (Boucher et al., 1981; Escribano et al., 1995; Kaestner et al., 1999; Tyler-McGowan and et al., 1999; Gouveia et al., 2003; Umbarila Barreto, 2007). Horses can rapidly increase the oxygen carrying capacity of the blood by increasing the hemoglobin concentration through splenic contraction (Kaestner et al., 1999; Muriel, 2016). 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 exercise, 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 (Lopez Chicharro, 2008).

In aerobic organisms, oxygen (O\textsubscript{2}) is essential for life as it is fundamental for energy metabolism. It can also be toxic and has been implicated in numerous diseases and degenerative conditions (Marx, 1985). Oxygen is used as the terminal oxidant in the mitochondrial respiratory chain, however, the presence of intracellular O\textsubscript{2} can result in the occurrence of redox reactions which may damage biomolecules (Imlay and Linn, 1986). Formation of free radicals during exercise depends on the intensity, frequency, duration and type. High levels of oxygen consumption during exercise have also been implicated as a contributing factor to oxidative stress (Ji, 1996; Ji, 1999; Williams et al., 2005; Kirschvink et al., 2008). Circulating erythrocytes are regularly exposed to stress conditions and are particularly vulnerable because they have no mechanisms of membrane repair or regenerative capacity (Cimen, 2008). During exercise, the oxidation of oxyhemoglobin to methemoglobin generates a great number of free radicals by release of O\textsubscript{2}, a condition that is directly related to the type of exercise and the oxygen needed by the tissues (Clemens and Waller, 1987). 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 (Deaton and Marlin, 2003). 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 cumenehydroperoxide (Tesoriere et al., 2001), tert-butyl hydroperoxide (t-BHP) (Mawatari and Murakami, 2001; Zou et al., 2001; Iglesias and Catala, 2005) and fatty acid hydroperoxides (Mawatari and Murakami, 1998; Udilova et al, 2003), which have been made from suspensions of red cell ghosts (Mawatari and Murakami, 1998, 2001; Tesoriere et al., 2001; Zou et al., 2001; Udilova et al., 2003; Iglesias and Catala, 2005; Muriel, 2016) and also from lysed cells (Sajewicz, 2010; Sajewicz et al. 2015; Savignone et al., 2016, Savignone and Palacios, 2017).

The aims of the present study were to analyze the oxidative alterations in the erythrocyte membrane of horses submitted to a high intensity exercise test by estimating the degree of peroxidation by chemiluminescence and evaluating 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 440 kg. They were housed in boxes all throughout the test period at the facilities of the Hospital School of the Faculty of Veterinary Sciences, National University of La Plata. 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 2.75 Mcal / 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 reproducing or simulating a maximum effort situation under controlled conditions. Animals were subjected to 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 14.6 m, width 9 m and height 10 m. 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.

The technical characteristics of the treadmill are:

- Length: 10.3 meters.
- Width: 3.64 meters.
- Floor to band height: 0.55 meters.
- Maximum height: 4.3 meters.
- Weight: 3770 kg.
- Total dimensions: 4 meters long and 1.2 meters wide.
- Machine speed: range 0-15 m/sec.
- Indice percentage: range 0 to 11%.
- 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 1 min preheating at a speed of 1.5 m/s and then 4 min at 4 m/s. This was followed by a 3% slope steps of 1 min with increasing intensities(5; 6; 7; 8; 9; 10; 11; 12; 13 m/sec, etc.) until fatigue. Then, the recovery phase was as follows: 4 and 1.5 m/s without slope during 4 and 1 min, respectively (Muriel, 2016). Three tests were performed on each animal 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. Thereafter, the catheter was connected to a flexible tube (both heparinized) long enough to gather blood sample without interrupting the exercise.

Blood samples were taken:

• Before exercise (T0 or rest)
• At fatigue (T1 or exercise)
• After the recovery phase (T2 or recovery) (Muriel, 2016).

Samples (5 ml 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 (1000 g 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 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. (1963). Briefly, packed, washed erythrocytes were lysed by adding 10 volume of 5 mM phosphate buffer pH 7.4 (at 4°C) while mixing and after leaving on ice for 30 min. Finally, the suspension was homogenized.

Peroxidation of erythrocyte analyzed by chemiluminescence

Peroxidation of erythrocyte lysates with final concentration of 0.25 mg/ml total hemoglobin was initiated by adding t-BHP (80 mM) 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 40 min period at 37°C, chemiluminescence was recorded as count per minute (cpm) every 10 min 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 (Wright et al., 1979).

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 (SSPS® Version 11).

RESULTS

Hematologic values

Table 1 and Figure 1 show the parameters of the red series evaluated for this study. Also, 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 8.44x10⁶ cell/μl (± 0.32), 11.65x10⁶ cells/μl (± 0.25) in T1 and 10.15x10⁶ 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 were 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).

Degree of peroxidation

The addition of t-BHP to the suspension of erythrocyte
**Table 1:** Hematologic values.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Extraction time</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
<th>Means± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hematocrit %</strong></td>
<td>T0</td>
<td>40.1</td>
<td>38.6</td>
<td>38.0</td>
<td>33.2</td>
<td>36.8</td>
<td>36.8</td>
<td>31.9</td>
<td>31.5</td>
<td>35.3</td>
<td>38.1</td>
<td>37.2</td>
<td>26.4</td>
<td>35.8</td>
<td>36.5</td>
<td>35.4±3.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>55.6</td>
<td>52.4</td>
<td>53.0</td>
<td>52.7</td>
<td>54.3</td>
<td>55.9</td>
<td>52.9</td>
<td>52.1</td>
<td>55.3</td>
<td>52.3</td>
<td>53.4</td>
<td>55.6</td>
<td>55.1</td>
<td>52.0</td>
<td>53.6±1.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>50.2</td>
<td>42.6</td>
<td>45.0</td>
<td>44.7</td>
<td>41.5</td>
<td>49.2</td>
<td>50.2</td>
<td>46.4</td>
<td>48.3</td>
<td>44.8</td>
<td>42.0</td>
<td>41.9</td>
<td>51.6</td>
<td>46.5</td>
<td>43.8</td>
<td>45.9±3.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>RBC 10&lt;sup&gt;6&lt;/sup&gt;cel/µl</strong></td>
<td>T0</td>
<td>10.0</td>
<td>10.1</td>
<td>9.5</td>
<td>7.3</td>
<td>7.7</td>
<td>7.2</td>
<td>8.4</td>
<td>7.0</td>
<td>6.7</td>
<td>7.4</td>
<td>10.3</td>
<td>9.7</td>
<td>9.2</td>
<td>8.5</td>
<td>7.7</td>
<td>8.4±1.2</td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>13.6</td>
<td>12.5</td>
<td>13.2</td>
<td>10.7</td>
<td>10.6</td>
<td>10.9</td>
<td>12.2</td>
<td>10.9</td>
<td>10.7</td>
<td>11.2</td>
<td>12.6</td>
<td>11.3</td>
<td>12.0</td>
<td>11.3</td>
<td>11.1</td>
<td>11.6±1.0</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>12.4</td>
<td>10.6</td>
<td>11.2</td>
<td>9.5</td>
<td>8.6</td>
<td>9.9</td>
<td>11.0</td>
<td>9.7</td>
<td>9.8</td>
<td>9.3</td>
<td>11.1</td>
<td>9.5</td>
<td>10.9</td>
<td>9.6</td>
<td>9.4</td>
<td>10.2±1.0</td>
</tr>
<tr>
<td><strong>Hemoglobin g/dl</strong></td>
<td>T0</td>
<td>13.3</td>
<td>12.9</td>
<td>13.2</td>
<td>12.4</td>
<td>13.5</td>
<td>12.5</td>
<td>13.2</td>
<td>11.8</td>
<td>11.9</td>
<td>13.1</td>
<td>12.8</td>
<td>13.6</td>
<td>13.5</td>
<td>13.8</td>
<td>12.5</td>
<td>12.9±0.6&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>18.3</td>
<td>16.4</td>
<td>17.1</td>
<td>17.7</td>
<td>18.6</td>
<td>19.1</td>
<td>18.9</td>
<td>18.1</td>
<td>18.7</td>
<td>19.7</td>
<td>16.8</td>
<td>17.2</td>
<td>18.6</td>
<td>18.5</td>
<td>17.6</td>
<td>18.1±1.9&lt;sup&gt;#&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>16.0</td>
<td>14.2</td>
<td>13.7</td>
<td>15.7</td>
<td>14.9</td>
<td>17.3</td>
<td>17.0</td>
<td>16.2</td>
<td>16.3</td>
<td>16.1</td>
<td>13.2</td>
<td>14.5</td>
<td>16.7</td>
<td>16.8</td>
<td>15.9</td>
<td>15.6±1.2&lt;sup&gt;#&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

ab; *#: means with different superscripts differ significantly at p<0.05.

**Figure 1:** Hematologic values.
The cells observed in this study, while plasma volume, and the fluid output to the extravascular space (Milne et al., 1976). 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 as compared with 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 (Williams et al., 2005).

**DISCUSSION**

The organism responds to physical demands are related to the type and duration of the activity. This response is manifested in several organic systems and the hematological system is not excepted, in particular the red blood cells studied in this work.

Literature has reported that hemoconcentration takes place at the beginning, and this is attributable to dehydration as a consequence of fluid loss through perspiration. It has also been reported, 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 (Bonilla, 2005). The increments in hemoglobin, hematocrit and red blood cells observed in this study, lead to hemoconcentration, determined by the mobilization of erythrocytes from the spleen (Persson, 1967), and the fluid output to the extravascular space (Milne et al., 1976). 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 as compared with 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 (Williams et al., 2005).

This is due to the production of reactive oxygen species, which cause cell and tissue damage (Clarkson and Thompson, 2000). 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 (Deaton and Marlin, 2003).

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

<table>
<thead>
<tr>
<th>Extraction time</th>
<th>Treatment</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
<th>Means± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>With t-BHP</td>
<td>260.1</td>
<td>370.9</td>
<td>281.7</td>
<td>271.2</td>
<td>401.2</td>
<td>246.1</td>
<td>400.5</td>
<td>425.0</td>
<td>277.9</td>
<td>495.5</td>
<td>401.2</td>
<td>252.5</td>
<td>401.2</td>
<td>256.2</td>
<td>342.8±10a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Without t-BHP</td>
<td>90.5</td>
<td>139.2</td>
<td>181.3</td>
<td>174.9</td>
<td>149.4</td>
<td>89.6</td>
<td>115.2</td>
<td>187.3</td>
<td>91.9</td>
<td>180.0</td>
<td>145.1</td>
<td>92.7</td>
<td>114.9</td>
<td>187.3</td>
<td>127.9</td>
<td>137.8±11b</td>
</tr>
<tr>
<td>T1</td>
<td>With t-BHP</td>
<td>250.4</td>
<td>262.9</td>
<td>193.0</td>
<td>184.2</td>
<td>256.2</td>
<td>174.9</td>
<td>250.7</td>
<td>315.5</td>
<td>251.5</td>
<td>308.7</td>
<td>271.2</td>
<td>251.5</td>
<td>174.9</td>
<td>262.9</td>
<td>225.5</td>
<td>242.3±11c</td>
</tr>
<tr>
<td></td>
<td>Without t-BHP</td>
<td>98.2</td>
<td>99.5</td>
<td>112.0</td>
<td>122.9</td>
<td>150.2</td>
<td>93.9</td>
<td>78.1</td>
<td>145.1</td>
<td>190.7</td>
<td>199.2</td>
<td>150.2</td>
<td>94.9</td>
<td>89.6</td>
<td>174.9</td>
<td>185.9</td>
<td>132.3±21b</td>
</tr>
<tr>
<td>T2</td>
<td>With t-BHP</td>
<td>234.9</td>
<td>275.4</td>
<td>141.3</td>
<td>210.2</td>
<td>243.2</td>
<td>224.2</td>
<td>234.3</td>
<td>278.7</td>
<td>240.3</td>
<td>231.0</td>
<td>250.7</td>
<td>234.7</td>
<td>234.3</td>
<td>260.1</td>
<td>250.7</td>
<td>236.3±11c</td>
</tr>
<tr>
<td></td>
<td>Without t-BHP</td>
<td>108.0</td>
<td>185.9</td>
<td>127.9</td>
<td>114.9</td>
<td>156.3</td>
<td>103.3</td>
<td>93.9</td>
<td>234.7</td>
<td>200.9</td>
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<td>99.5</td>
<td>105.3</td>
<td>193.0</td>
<td>210.2</td>
<td>114.9</td>
<td>143.0±8b</td>
</tr>
</tbody>
</table>

abc: means with different superscripts differ significantly at p<0.05.
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 (Murakami and Mawatari, 2003), and that erythrocytes have mechanisms for protection against oxidative damage, including catalase (Agar et al., 1986), superoxide dismutase (Fee and Teitelbaum, 1972), and low molecular weight antioxidants such as ascorbate (Meister, 1994), we must consider that the existence of changes in erythrocyte membranes, evaluated by peroxidation, is a consequence of physical exercise. The results clearly suggest the pro-oxidizing environment prevailing in the blood during high-intensity exercise, probably associated with the release of ROS induced by such circumstance.

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 was observed. The latter may be attributed 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.

**ACKNOWLEDGEMENT**

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