Gender differences in exercise – induced intravascular haemolysis during race training in thoroughbred horses

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1. Introduction

Exercise related intravascular haemolysis is a pathophysiological condition, recognized in both human and equine athletes. In human, it is postulated as one of the causes of “sport anemia” and iron deficiency (Telford et al., 2003; Beneke et al., 2005; Peeling et al., 2009). It was described over 120 years ago in men after long-distance march and since that time is has been widely reported after swimming, cycling, rowing, weight training and resistance training (Selby and Eichner, 1986; Schobersberger et al., 1990; Beneke et al., 2005; Peeling et al., 2008).

Intravascular haemolysis has been determined in well-trained endurance runners and triathletes as well as in untrained individuals participating training programs (Beneke et al., 2005; Peeling et al., 2009), however, the half-life of erythrocytes in male and female athletes is shorter than in non-athletic subjects (Weight et al., 1991). In well-trained endurance athletes, the customary training sessions are followed by hemolytic episodes and the cumulative effect has been shown (Peeling et al., 2009).

Intravascular haemolysis is indicated by an increase of free plasma haemoglobin accompanied by decrease of serum haptoglobin level (Sakurada and Tanaka, 1996; Malczewska et al., 2000) and the presence of hemoglobinific pigment in urine (Schott et al., 1995).

Changes in haptoglobin level reflect intravascular haemolysis as haptoglobin binds free plasma haemoglobin, so that prevents its excretion in the urine and makes iron and globin able to recycle in the liver (Hanzawa et al., 2002; Petersen et al., 2004). In humans, this parameter is postulated as a good biochemical marker for the monitoring of an athlete’s status (Sobiech, 2000). In horses serum haptoglobin level has been proved to be a useful indicator of infection, inflammation and haemolytic disease (Mills et al., 1998; Petersen et al., 2004).

Intravascular haemolysis after exertion has been reported also in horses, however the literature is more limited and presented results vary among authors. Evidences confirming such condition has been found after endurance rides (Murakami, 1974), race (Pellegrini Masini et al., 2003) and treadmill exercise of various intensity (Schott et al., 1995; Inoue et al., 2005). The authors reported increases in plasma free haemoglobin levels (Pellegrini Masini et al., 2003; Inoue et al., 2005), decreases in plasma haptoglobin levels (Pellegrini Masini et al., 2003), serum iron concentration (Inoue et al., 2005) and transient haematuria (Schott et al., 1995). It is interesting, that intravascular haemolysis occurred after treadmill exercise at speed eliciting 60% of the maximal oxygen consumption (Schott et al., 1995) and after treadmill exercise of moderate intensity (Inoue et al., 2005). These observations may suggest, that common training sessions in race horses may also cause an intravascular haemolysis. Changes in haptoglobin level throughout training season were investigated by Willett and Blackmore.
They found no variations and suggested, that measurements of haptoglobin is useful in the detection of frank haemolysis, but insufficiently sensitive to detect mild red cell destruction (Willett and Blackmore, 1979). However, they examined horses four times during training season and did not investigated the differences between the values before and after exercise. Exercise-induced intravascular haemolysis has been confirmed under various conditions in stallions (Inoue et al., 2005), mares (Schott et al., 1995) and mixed population (Hanzawa et al., 2002). In equine sports stallions and mares compete together. It is generally believed, that exercise-induced haematological changes only slightly differ between genders. This is true taking into consideration only basic haematological values, but significant differences in more specific parameters, including non-specific immune response in stallions and mares have been reported (Escribano et al., 2008). The parameters indicating intravascular haemolysis have never been compared between genders. However, it has been shown in rodents that cation-osmotic haemolysis in high ionic strength media is significantly higher in male (Marossy et al., 2004). Thus, the aim of this study was to investigate (1) if common training sessions in race horses result in exercise-induced intravascular haemolysis and (2) whether this condition differs in male and female athletic horses.

2. Materials and methods

2.1. Horses and training

Twenty-four thoroughbred horses in regular training, 12 mares and 12 stallions, ranging from 3 to 6 years of age, weighing 520 ± 50 kg, housed in the same training center (Slużewiec Race Track in Warsaw) were included in the study. Horses were privately owned and were prepared to participate in racing events by the same trainer. The animals were selected on the basis of clinical examination performed by qualified veterinarian and haematological analysis to exclude pathological conditions, and on the basis of similar racing performance, as recorded from the previous year’s athletic merits. The procedures have been approved by Local Ethics Committee and the owners of the horses. All the horses were dewormed and vaccinated at similar time, housed in the same environment and fed the diet (hay and oat) that maintain the requirements for exercising horses. Salt and water were available ad libitum.

Stallions and mares trained together in mixed group with the same intensity at Slużewiec Race Track in Warsaw and were examined at the same days, four times, with 4 weeks intervals (in March, April, May and June), before and after training sessions (Table 1) including 10 min of walking, 5 min of trotting, 7 min of cantering and 30 min of exercise on horse walker. The training was monitored using heart rate monitors RS800 on Polar Equine Wearlink (W.I.N.D (POLAR EQUINE) and G3 GPS with sensor.

2.2. Blood samples

Blood samples were obtained by jugular venipuncture before and immediately after each training session. Samples were aspirated into 20 ml syringe and immediately transferred into EDTA tubes for haematological tests, heparinized tubes for plasma hemoglobin measurements and into plain tubes for serum analyses. Lactate concentrations were determined immediately by ejecting a drop of full blood onto single-use lactate strip (Accusport, Roche). The EDTA tubes were kept in refrigerator (+4°C) and analyzed within 6 h after collection. Routine hematological parameters: haematocrit (HCT), haemoglobin concentration (HGB) and the number of red blood cells (RBC) were counted with an automated hematology analyzer (Abacus). Heparinized and plain tubes were promptly centrifuged and plasma/serum samples immediately frozen at −20°C. Free haemoglobin levels were determined in plasma samples spectrophotometrically, according to Kahn’s method (Kahn et al., 1981). Serum samples were used for the measurements of total protein and haptoglobin levels. Total protein levels were determined by using total protein (Biuret) reagent set (POINTE SCIENTIFIC). Serum haptoglobin was measured colorimetrically using PHASE haptoglobin assay (TRIDELTA LTD). The method is based on the preservation of peroxidase activity of bound haemoglobin at low pH. Thus, the amount of haptoglobin present in the specimen is directly proportional to the preserved peroxidase activity of haemoglobin (inhibited at low pH in case of free haemoglobin).

Plasma haemoglobin and serum haptoglobin levels were analyzed after correction to avoid the influence of haemococoncentration. The values obtained after exertion were recalculated taking into account the changes in total protein concentrations according to the following formulation:

\[ H_{corr} = H \times \frac{TP_1}{TP_2}, \]

where 

- \( H_{corr} \) = corrected serum haptoglobin or plasma haemoglobin concentration, 
- \( H \) = serum haptoglobin or plasma haemoglobin concentration determined after exertion, 
- \( TP_1 \) = total protein level before exertion, 
- \( TP_2 \) = total protein level after exertion.

2.3. Statistical analysis

Statistical procedures, means and standard errors of mean were computed using the STATISTICA 6.0 for Windows. Results are expressed as mean ± standard errors of mean (SEM). Statistical comparisons between the results obtained in stallions and mares were made with Mann–Whitney U test. The results before and after

<table>
<thead>
<tr>
<th>Training session</th>
<th>Maximal heart rate (beats/min)</th>
<th>Average heart rate (beats/min)</th>
<th>Maximal speed (km/h)</th>
<th>Lactate before training (mmol/l)</th>
<th>Lactate after training (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st (March)</td>
<td>Mares 196.67 ± 2.52</td>
<td>104.58 ± 3.22</td>
<td>41.7 ± 3.05</td>
<td>1.33 ± 0.21</td>
<td>3.86 ± 0.87*</td>
</tr>
<tr>
<td></td>
<td>Stallions 196.78 ± 4.51</td>
<td>109.92 ± 2.54</td>
<td>41.88 ± 4.09</td>
<td>1.77 ± 0.17</td>
<td>4.90 ± 1.54*</td>
</tr>
<tr>
<td>2nd (April)</td>
<td>Mares 204.5 ± 4.83</td>
<td>110.22 ± 5.75</td>
<td>49.6 ± 3.5</td>
<td>1.30 ± 0.26</td>
<td>5.93 ± 1.21*</td>
</tr>
<tr>
<td></td>
<td>Stallions 195.42 ± 3.05</td>
<td>102.17 ± 2.07</td>
<td>44.09 ± 2.81</td>
<td>1.07 ± 0.08</td>
<td>4.60 ± 1.19*</td>
</tr>
<tr>
<td>3rd (May)</td>
<td>Mares 199.09 ± 3.80</td>
<td>104.25 ± 3.71</td>
<td>40.78 ± 3.95</td>
<td>1.36 ± 0.16</td>
<td>6.28 ± 1.48*</td>
</tr>
<tr>
<td></td>
<td>Stallions 196.60 ± 5.88</td>
<td>105.7 ± 4.78</td>
<td>47.78 ± 2.81</td>
<td>1.07 ± 0.08</td>
<td>4.6 ± 1.19*</td>
</tr>
<tr>
<td>4th (June)</td>
<td>Mares 199.00 ± 5.55</td>
<td>105.29 ± 5.29</td>
<td>49.49 ± 1.19</td>
<td>1.38 ± 0.16</td>
<td>6.68 ± 0.92*</td>
</tr>
<tr>
<td></td>
<td>Stallions 204.8 ± 6.85</td>
<td>103.00 ± 5.49</td>
<td>48.9 ± 2.76</td>
<td>1.60 ± 0.18</td>
<td>5.08 ± 0.72*</td>
</tr>
</tbody>
</table>

Differences between the values before and after exercise: *p < 0.001.
Training sessions in each gender were compared with Wilcoxon test; \( p \leq 0.05 \) was considered significant.

3. Results

Mean and maximum heart rate, maximum speed and lactate levels did not differ between stallions and mares (Table 1), thus confirmed, that the exertion was similar and relatively high in both genders. All haematological parameters (Table 2) before and after exercise varied within normal ranges for equine species (Hinchcliff et al., 2004; Smith, 2008). In both, mares and stallions, RBC, HCT, HBG and lactate concentrations increased in response to exercise \( (p \leq 0.001) \), however there were no significant differences between genders. The significant increase in total protein concentration was detected only after the 3rd training session in stallions and after the 4th training session in mares; no gender differences were recorded.

Plasma haemoglobin level before training sessions did not differ between stallions and mares. In mares, the significant increases were observed after 3rd and 4th training sessions (Fig 1), but no such changes were detected in stallions (Table 2). Serum haptoglobin concentrations were higher in mares than in stallions before and after all training sessions (Fig. 2a and b). In stallions it did not change significantly after the exertion, but in mares it was decreased after 4th training session (Fig. 1).

### Table 2

Hematological parameters before and after training sessions; 12 male and 12 female thoroughbred horses, 3–6 years of age.

<table>
<thead>
<tr>
<th>Training sessions</th>
<th>HCT (%)</th>
<th>HGB (g/dl)</th>
<th>RBC ((10^6)/\mu l)</th>
<th>Tot Pr (g/l)</th>
<th>pHGB (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stallions</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st (March) Before</td>
<td>44.94 ± 1.23</td>
<td>15.08 ± 0.42</td>
<td>10.60 ± 0.30</td>
<td>65.16 ± 1.81</td>
<td>0.63 ± 0.38</td>
</tr>
<tr>
<td>After</td>
<td>59.54 ± 1.50(^a)</td>
<td>18.58 ± 0.34(^b)</td>
<td>13.97 ± 0.41(^c)</td>
<td>67.70 ± 2.12</td>
<td>1.04 ± 0.46</td>
</tr>
<tr>
<td>2nd (April) Before</td>
<td>41.90 ± 0.97</td>
<td>14.23 ± 0.29</td>
<td>10.04 ± 0.22</td>
<td>65.94 ± 2.83</td>
<td>0.87 ± 0.45</td>
</tr>
<tr>
<td>After</td>
<td>58.89 ± 2.00(^a)</td>
<td>18.39 ± 0.49(^b)</td>
<td>14.00 ± 0.45(^c)</td>
<td>68.23 ± 1.32</td>
<td>0.99 ± 0.28</td>
</tr>
<tr>
<td>3rd (May) Before</td>
<td>43.04 ± 1.20</td>
<td>14.46 ± 0.38</td>
<td>10.41 ± 0.31</td>
<td>65.36 ± 0.74</td>
<td>0.56 ± 0.15</td>
</tr>
<tr>
<td>After</td>
<td>56.27 ± 2.00(^a)</td>
<td>18.05 ± 0.40(^b)</td>
<td>13.64 ± 0.48(^c)</td>
<td>69.88 ± 1.09(^b)</td>
<td>0.75 ± 0.11</td>
</tr>
<tr>
<td>4th (June) Before</td>
<td>42.69 ± 0.94</td>
<td>15.06 ± 0.23</td>
<td>10.51 ± 0.24</td>
<td>65.41 ± 1.03</td>
<td>0.74 ± 0.16</td>
</tr>
<tr>
<td>After</td>
<td>57.81 ± 1.66(^a)</td>
<td>18.97 ± 0.43(^b)</td>
<td>13.97 ± 0.49(^c)</td>
<td>66.83 ± 1.42</td>
<td>0.99 ± 0.22</td>
</tr>
<tr>
<td><strong>Mares</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st (March) Before</td>
<td>43.21 ± 2.06</td>
<td>14.64 ± 0.58</td>
<td>10.58 ± 0.45</td>
<td>64.36 ± 2.65</td>
<td>0.58 ± 0.24</td>
</tr>
<tr>
<td>After</td>
<td>51.65 ± 1.31(^b)</td>
<td>17.10 ± 0.29(^c)</td>
<td>12.80 ± 0.23(^a)</td>
<td>68.62 ± 2.45</td>
<td>0.81 ± 0.12</td>
</tr>
<tr>
<td>2nd (April) Before</td>
<td>40.63 ± 1.20</td>
<td>13.72 ± 0.33</td>
<td>9.99 ± 0.30</td>
<td>63.35 ± 2.14</td>
<td>0.84 ± 0.45</td>
</tr>
<tr>
<td>After</td>
<td>56.95 ± 1.37(^b)</td>
<td>18.07 ± 0.29(^c)</td>
<td>13.89 ± 0.30(^a)</td>
<td>64.68 ± 1.95</td>
<td>0.95 ± 0.14</td>
</tr>
<tr>
<td>3rd (May) Before</td>
<td>41.74 ± 0.97</td>
<td>14.08 ± 0.26</td>
<td>10.43 ± 0.25</td>
<td>64.53 ± 0.79</td>
<td>0.58 ± 0.14</td>
</tr>
<tr>
<td>After</td>
<td>55.13 ± 1.93(^a)</td>
<td>17.76 ± 0.44(^b)</td>
<td>13.63 ± 0.40(^c)</td>
<td>65.43 ± 0.95</td>
<td>0.91 ± 0.19(^a)</td>
</tr>
<tr>
<td>4th (June) Before</td>
<td>41.77 ± 0.75</td>
<td>14.58 ± 0.20</td>
<td>10.58 ± 0.22</td>
<td>62.09 ± 0.51</td>
<td>0.63 ± 0.15</td>
</tr>
<tr>
<td>After</td>
<td>55.95 ± 1.59(^b)</td>
<td>18.39 ± 0.36(^c)</td>
<td>14.01 ± 0.38(^a)</td>
<td>66.80 ± 1.12(^b)</td>
<td>1.44 ± 0.19(^a)</td>
</tr>
</tbody>
</table>

HCT – hematocrit; HGB – haemoglobin concentration; RBC – number of red blood cells; Tot Pr – total protein concentration; pHGB – plasma haemoglobin.

Differences between the values before and after exercise: \(^a\)\( p \leq 0.05; \(^b\)\( p \leq 0.01; \(^c\)\( p \leq 0.001.\)

Fig. 1. Plasma haemoglobin and serum haptoglobin levels in mares before and after training sessions. Differences between plasma haemoglobin concentrations before and after exercise: \(^* p \leq 0.05.\) Differences between serum haptoglobin concentrations before and after exercise: \(^* p \leq 0.05.\)
In response to exertion, splenic erythrocytes are released due to increased bovine liver parenchymal cells in vitro (Higuchi et al., 2006). Haptoglobin in horses and cattle other relations has been reported.

Exercise-induced haemolysis in mares has been reported by Schott et al. It was recognized on the basis of pigmenturia after treadmill exercise at 60 and 95% V\textsubscript{O\textsubscript{max}} (Schott et al., 1995). Intravascular haemolysis in athletic horses results from increased fragility of erythrocytes (Hanzawa et al., 1999; Hanzawa and Watanabe, 2000) due to frequent accumulation in the spleen (Hanzawa et al., 1999, 2002). Anaerobic nature of the exercise also promotes the increase in osmotic fragility of red cells, while aerobic exertion cause the opposite effect (Hanzawa et al., 1992, 1995, 2002).
1996; Smith et al., 1989). During anaerobic exercise the fragility of erythrocytes increases progressively with the running velocity (Hanzawa and Watanabe, 2000). It is also related with the decrease of blood pH resulting from the increases of carbon dioxide partial pressure and lactate concentration (Carlson, 1995; Kronfeld et al., 1995; Hanzawa and Watanabe, 2000). In our study the exertion was the same in all the horses and relatively high, but intravascular haemolysis was observed only in mares. This fact, however, seems to be of limited clinical importance due to the higher serum haptoglobin levels before the exercise in mares and no relation to athletic merits, moreover, no evidence for cumulative effect was noted.

Gender differences in sport horses receive little attention due to similar athletic capacity and basic haematological parameters in stallions and mares. Mixed populations are analyzed frequently. However, sexual dimorphism exists, as it has been proved in the context of non-specific immune response (Escribano et al., 2008). The authors reported slight differences in the parameters of non-specific response and different correlations with catecholamine specific response and different correlations with catecholamine

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