Reticulocyte changes after experimental anemia and erythropoietin treatment of horses

C. Cooper,1 W. Sears,2 and D. Bienzle1

Departments of Pathobiology, and 2Population Medicine, University of Guelph, Guelph, Ontario, Canada

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Cooper, C., W. Sears, and D. Bienzle. Reticulocyte changes after experimental anemia and erythropoietin treatment of horses. J Appl Physiol 99: 915–921, 2005; doi:10.1152/japplphysiol.00438.2005.—Availability of recombinant human erythropoietin (EPO) has facilitated use to enhance red blood cell production, and therefore aerobic performance, in human and equine athletes. Recombinant human EPO promotes growth and differentiation of equine erythroid precursor cells, but in some horses repeat administration induces immune interference with endogenous EPO resulting in fatal anemia. Although blood reticulocyte parameters acquire unique changes in humans treated with EPO, with manual enumeration methods, horses were not considered to release reticulocytes from the bone marrow into circulation, even under severe erythropoietic stress. The goals of this study were to determine whether reticulocytes could be detected and characterized in horses that are anemic or have been treated with EPO using a modern hematology analyzer. Anemia was induced in six horses by removal of 30 ml of blood/kg of body wt over 24 h. After 28 days, the horses were treated twice with 55 U/kg of EPO (Eprex), and after 65 days they were treated thrice with 73 U/kg of EPO. Blood samples were analyzed with the ADVIA120 instrument every 3–5 days and bone marrow samples 7 days after anemia and EPO treatments. Analysis of blood reticulocyte parameters by ANOVA in a randomized complete block design determined that anemia and EPO induced significant (P ≤ 0.05) increases in red cell distribution width and reticulocyte mean cell volume. Parameters changed only after EPO treatment were cellular hemoglobin concentration mean, mean cell volume, reticulocyte concentration, proportion of macrocytic reticulocytes, and reticulocyte cellular hemoglobin. These findings indicate that horses under erythropoietic stress and after EPO treatment release reticulocytes with unique characteristics into circulation.

Erythropoietin (EPO) is a cytokine released in response to hypoxia, which promotes erythroid precursor cell growth and differentiation in the bone marrow. EPO rescues erythroid progenitors at the colony-forming unit erythroid to erythroblast stages from apoptosis, and increases the production of reticulocytes and the circulating RBC mass (11). Because EPO activity is highly specific for erythroid cells, recombinant human EPO (rhEPO) is used extensively to treat people with anemia due to chronic renal disease and bone marrow suppression from cancer therapy and to limit blood transfusions (1, 6). The ability to increase RBC mass, oxygen-carrying capacity, and muscle function, the relative lack of undesirable side effects, the very short half-life in circulation, and difficulty with distinguishing endogenous from recombinant cytokine in the laboratory are attractive attributes for the illicit use of rhEPO as a performance-enhancing drug (21). Precise figures are unavailable, but it has been estimated that high-performing athletes widely abuse EPO (27). Deleterious side effects of EPO overadministration may result in polycythemia, leading to excessive blood viscosity and potential death from cerebral thromboembolism (27). EPO is highly conserved among mammalian species; human and equine EPO have 83.8% identity at the amino acid level (24). This high degree of sequence similarity accounts for the cross-species activity of EPO and allows for recognition of the EPO glycoprotein of many species with polyclonal antibodies against human EPO (12). In horses, akin to humans, administration of rhEPO increases the RBC mass and oxygenation and has been used to enhance racing performance (10, 19, 28). Subtle differences in the glycosylation of rhEPO compared with endogenous human EPO can induce antibody formation and pure red cell aplasia in humans due to inhibition of endogenous EPO production (1). Similarly, horses treated with rhEPO have developed immune responses and fatal pure red cell aplasia against undefined components of rhEPO (22, 25, 37).

Illicit EPO use in human athletes may be detected indirectly by analysis of specific reticulocyte parameters (26) or directly in urine by chromatographic and other high-resolution techniques (30). In humans, the changes in reticulocyte parameters resulting from EPO administration were distinct from those of other hematological conditions but common to all forms of the recombinant cytokine (20, 21). An ELISA test is commercially available to detect EPO levels in human urine, although the assay is not specific for rhEPO. Because of the high degree of sequence similarity between human and horses, an ELISA test may also have some utility in horses to detect rhEPO use. Because EPO is highly conserved among mammalian species, a polyclonal antibody raised against human EPO should have some cross-reactivity with equine EPO. In a study of urine samples from the Kentucky horse track, 8 out of 10 samples collected from nonperfomers were negative for EPO. However, 2 out of 3 samples collected from horses with anemia were positive for EPO. These results indicate that an EPO assay may be useful as an anti-doping test for horses. The simplicity and cost-effectiveness of an EPO assay are attractive attributes for an anti-doping test, but the need for cross-reactivity with equine EPO further complicates the development of an EPO test for horses.
available to detect antibodies against rhEPO in equine serum (G. Maylin, Equine Drug Testing, Ithaca, NY), but data detailing assay performance are unavailable. The goals of this study were to determine 1) whether a sensitive hematology analyzer can detect reticulocytes in horses after experimental induction of anemia and after EPO treatment, and 2) whether different causes of altered erythropoiesis induce unique changes in reticulocytes.

MATERIALS AND METHODS

Animals and study design. Six horses ranging in age from 5 to 15 yr were available for study: four Standardbred horses, one Thoroughbred horse, and one Canadian horse. Four horses were geldings, and two were mares; their weight ranged from 420 to 545 kg. All horses were free of abnormalities on physical and hematomatological examination, did not receive medications or donate blood during 6 wk before study, and were subject to routine vaccination and parasite-control programs. The horses were maintained on pasture with water access during the study. This study was approved by the institutional Animal Care Review Committee in accordance with guidelines provided by the Canadian Council for Animal Care.

From each animal, seven blood samples were analyzed over 28 days for determination of baseline parameters. Then, animals were rendered anemic by phlebotomy and allowed to recover for 28 days. Next, they were treated twice with a low dose of EPO, allowed to recover for ~28 days, and then treated thrice with a higher dose of EPO. Blood samples throughout the study were analyzed every 3–5 days, and new treatments were initiated once the Hct had returned to EPO. Three smears of bone marrow were prepared on glass slides and stained with Wright’s stain, and an aliquot was analyzed in the ADVIA120 hematometer instrument as described above. The proportion of granulocytic to erythrocytic precursor cells in the bone marrow (M/E ratio) was determined by 500-cell differential counting of Wright’s stained bone marrow smears. Bone marrow samples were collected on day −7 and 7 days after induction of anemia and EPO treatments.

EPO and iron treatment. Each horse was administered rhEPO (epoietin alfa, Eprex, Janssen-Ortho, Toronto, Ontario, Canada) intravenously at two different dosages: two doses of 55 U/kg, 48 h apart, starting on day 30; and three doses of 73 U/kg, 48 h apart, starting on day 64 (Fig. 1). In addition, each horse received three doses of 500 mg of intravenous iron (Hippiron 1000, Bioniche Animal Health, Belleville, Ontario, Canada) during the second series of EPO treatments.

Statistical analysis. Hematological responses were recorded over time; therefore, potential autocorrelation among the residuals had to be accounted for. For the blood parameters, the summary statistic approach was used (8). Because it was primarily of interest to assess response change, means of the first 10 and 28 days were computed, except in baseline period, when all data were used to compute the mean. Then, these means were treated as the response observations and analyzed using a general linear mixed model in the form of a randomized complete block design, with treatment the primary factor and horse as the random blocking factor. To help choose the autocorrelation structure, Akaike information criterion was employed. To validate the model and assess whether the ANOVA assumptions were met, residual plots were constructed, plotting the residuals against predicted values and factor codes. Formal tests of normality of the residuals (Shapiro-Wilk, Anderson-Darling, Kolmogorov-Smirnov, and Cramer-von Mises) were also applied. The bone marrow measures were simpler since only one response per treatment was obtained; thus, there was no need to compute summary statistics. The model and analyses were otherwise the same as for the blood measures. For both bone marrow and blood analyses, Dunnett’s test was applied to comparisons between the treatments and baseline to determine the presence and nature of differences and the 95% confidence intervals on the differences. The commercial statistics package SAS 8.2 (Cary, NC) was used for analyses, primarily employing Proc SUMMARY and Proc MIXED.

RESULTS

Animals and procedures. No adverse effects attributable to induction of anemia or treatment with EPO were observed. All horses had normal hematological findings 4 mo after conclusion of the study, indicating that an immune response interfering with endogenous equine EPO production or function was not induced. ELISA results for rhEPO antibodies were negative for all samples (data not shown).

Blood parameters. The parameters selected for statistical analysis after automated hematological examination were Hct, red cell distribution width (RDW), cellular hemoglobin concentration mean (CHCM), mean cell volume (MCV), reticulocyte concentration (Retic), mean reticulocyte cell volume (MCRV), macrocytic reticulocyte concentration (MacroRetic) and reticulocyte cellular hemoglobin (CHr). At baseline, assessment of instrument precision yielded coefficients of variation of <10% for each parameter except Retic (23.9%) and MacroRetic (20.9%). Six days after induction of anemia, coefficients of variation ranged from 0.9 to 9.0% for all parameters. Residual analyses revealed no irregular distribution of the data, so all ANOVA assumptions appeared to be adequately met.
Data analyzed over each entire 28- to 30-day treatment period showed that phlebotomy induced a significant decrease in the Hct (0.06 – 0.12 liter/liter; Fig. 2) and resulted in significant increases in RDW, CHCM, MCV, MCVr, and CHr (Table 1 and Fig. 2). Treatment with EPO induced greater changes in the RDW, CHCM, and CHr, and resulted in increases in MacroRetic, but did not significantly increase Hct. Only the higher dose of EPO resulted

Table 1. Changes in blood RBC parameters over 28 days after induction of anemia and EPO treatment

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline Mean ± SE</th>
<th>Anemia Mean ± SE</th>
<th>EPO-L Mean ± SE</th>
<th>EPO-H Mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hct, liter/liter</td>
<td>0.37 ± 0.01</td>
<td>0.31 ± 0.01</td>
<td>0.38 ± 0.01</td>
<td>0.39 ± 0.01</td>
</tr>
<tr>
<td>RDW, %</td>
<td>17.36 ± 0.19</td>
<td>17.96 ± 0.25</td>
<td>18.34 ± 0.20</td>
<td>20.16 ± 0.27</td>
</tr>
<tr>
<td>CHCM, g/l</td>
<td>357.62 ± 0.49</td>
<td>359.26 ± 0.49</td>
<td>366.10 ± 0.49</td>
<td>371.06 ± 0.49</td>
</tr>
<tr>
<td>MCV, fl</td>
<td>47.12 ± 0.92</td>
<td>48.07 ± 0.92</td>
<td>48.23 ± 0.92</td>
<td>47.38 ± 0.92</td>
</tr>
<tr>
<td>Retic, 10⁹/l</td>
<td>2.97 ± 0.56</td>
<td>4.01 ± 0.56</td>
<td>3.42 ± 0.56</td>
<td>5.51 ± 0.56</td>
</tr>
<tr>
<td>MCVr, fl</td>
<td>61.17 ± 1.22</td>
<td>65.05 ± 0.77</td>
<td>68.69 ± 1.06</td>
<td>69.71 ± 0.56</td>
</tr>
<tr>
<td>MacroRetic, %</td>
<td>24.17 ± 2.85</td>
<td>29.55 ± 1.54</td>
<td>36.62 ± 2.35</td>
<td>40.89 ± 1.70</td>
</tr>
<tr>
<td>CHr, pg</td>
<td>20.25 ± 0.24</td>
<td>21.36 ± 0.24</td>
<td>22.59 ± 0.24</td>
<td>23.41 ± 0.24</td>
</tr>
</tbody>
</table>

RBC, red blood cell; EPO, erythropoietin; CI, confidence interval; Hct, hematocrit; RDW, red cell distribution width; CHCM, cellular hemoglobin concentration mean; MCV, mean cell volume; Retic, reticulocyte concentration; MCVr, reticulocyte mean cell volume; MacroRetic, macrocytic reticulocyte concentration; reticulocyte cellular hemoglobin, EPO-L, 2 doses of 55 U/kg; EPO-H, 3 doses of 73 U/kg.
in a significant increase in the number of reticulocytes (Table 1).

When changes in RBC parameters over the first 10 days (3 measurements) after high-dose EPO treatment were compared with those at baseline, significant differences in all parameters except MCV were identified (Table 2). Differences were not observed in the white blood cell and platelet concentrations. Serum iron concentration and total iron binding capacity were within equine reference limits throughout the study (data not shown).

Bone marrow parameters. Anti-coagulated bone marrow samples were aspirated from sternebrae at baseline, 1 wk after phlebotomy, and after each EPO treatment. RBC parameters in the bone marrow, which included a large number of reticulocytes, were very similar among horses (Fig. 3). Consistent with a regenerative response to anemia, MCV, Retic, MCVr, and CHr increased dramatically after phlebotomy, then returned to approximately baseline levels on day 40, and increased again after treatment with the high dose of EPO (Table 3, Fig. 3). Bone marrow Hct and reticulocyte cellular hemoglobin concentration mean decreased after phlebotomy and increased with each EPO treatment. The nucleated cell count (NCC)/Hct increased after anemia but not after EPO treatment. The M/E ratio in the bone marrow of horses decreased from a mean of 0.75 at baseline to 0.48 at 7 days after anemia, and ranged from 0.65 to 0.36 after EPO treatment (data not shown).

DISCUSSION

Factors that determine the release of reticulocytes from the bone marrow space across the bone marrow-blood barrier into the circulating blood compartment are largely unknown. Species differences controlling this transition are striking: rodents and pigs typically have numerous circulating reticulocytes, whereas humans, dogs, and cats have low numbers, and horses have none or very few. These unique species characteristics were identified with older methods of incubating blood with "vital" stains (such as new methylene blue) that are specific for nucleic acids, followed by manual enumeration of RBC containing stain precipitate on smears (5, 9). Newer hematology analyzers, including the Advia120, determine the proportion of reticulocytes among cells with light absorbance and scatter characteristics typical of RBC by automated detection of cells that fluoresce after incubation with a cationic nucleic acid stain, oxazine 750 (31, 35). Although the manual methods typically determine the proportion of reticulocytes per 1,000 RBC, automated methods identify the proportion of nucleic

<table>
<thead>
<tr>
<th>Parameter</th>
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<th>Anemia</th>
<th>EPO-L</th>
<th>EPO-H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hct, liter/liter</td>
<td>0.37±0.01</td>
<td>0.28±0.01</td>
<td>0.37±0.01</td>
<td>0.40±0.01</td>
</tr>
<tr>
<td>RDW, %</td>
<td>17.36±0.20</td>
<td>17.50±0.20</td>
<td>18.15±0.20</td>
<td>20.06±0.20</td>
</tr>
<tr>
<td>CHCM, g/l</td>
<td>357.62±0.46</td>
<td>358.89±0.64</td>
<td>369.44±0.47</td>
<td>396.44±0.47</td>
</tr>
<tr>
<td>MCV, fl</td>
<td>47.12±0.91</td>
<td>48.073±0.91</td>
<td>47.77±0.91</td>
<td>47.77±0.91</td>
</tr>
<tr>
<td>Retic, 10^7/L</td>
<td>2.97±0.25</td>
<td>2.45±0.99</td>
<td>2.38±0.45</td>
<td>2.57±0.80</td>
</tr>
<tr>
<td>MCVr, fl</td>
<td>61.17±0.85</td>
<td>62.13±0.85</td>
<td>70.87±2.23</td>
<td>75.99±1.46</td>
</tr>
<tr>
<td>MacroRetic, %</td>
<td>24.17±2.28</td>
<td>23.50±1.68</td>
<td>43.56±5.29</td>
<td>52.30±3.04</td>
</tr>
<tr>
<td>CHr, pg</td>
<td>20.25±0.25</td>
<td>20.53±0.31</td>
<td>23.12±0.65</td>
<td>25.13±0.57</td>
</tr>
</tbody>
</table>

Fig. 3. Changes in bone marrow red blood cell parameters of individual horses after anemia and EPO treatment. Each line represents an individual animal. CHCMr, reticulocyte cellular hemoglobin concentration mean. *Mean is different from baseline (P < 0.05).
acids-containing cells in >40,000 RBC (D. Zelmanovic, personal communication), depending on the RBC density of the sample. This has yielded more sensitive and reproducible estimates of reticulocyte numbers in human blood samples (13, 33). Furthermore, direct measurement of the size and hemoglobin concentration of a large number of individual RBC and reticulocytes allows for automated analysis of variation in these parameters, which was not possible with manual reticulocyte determinations and only to a limited extent in instruments using electrical impedance (14, 33). For these reasons, it was of interest to reexamine the traditional paradigm that horses do not release reticulocytes by exposure to hypoxic stress and treatment with rhEPO.

Anemia was induced by removal of 30 ml of blood/kg body wt over 24 h. The adult equine blood volume has been estimated at 72 ml/kg (29); therefore, the phlebotomy removed ~42% of the horses’ blood volume. Remarkably, only a transient decrease in Hct resulted; the maximal decrease observed was 29.5%. This relative lack of anemia after profound blood loss was previously reported (15, 18, 23) and is likely attributable to the great RBC storage and release capacity of the equine spleen. Anemia resulted in few significant changes in RBC or reticulocytes during the first 10 days, but if assessed after 28 days, the RDW, MCV, MCVr, and CHr increased. Increases first in RDW, and later in MCV, are well-recognized responses to anemia in horses (15, 16, 19, 23). CHr has not previously been measured in anemic horses, but the amount of hemoglobin per reticulocyte is increased in regenerative anemia of iron-replete humans, whereas the relative hemoglobin concentration (CHCM) of all RBC is typically decreased (2). Horses in this study had adequate serum iron throughout the study, which may have contributed to increased hemoglobin content in reticulocytes and saturation in RBC. Although the number of reticulocytes increased above baseline after induction of anemia, there was marked individual variation, which precluded identifying significant changes in the group. Variation in the reticulocyte response could not be attributed to age, gender, or breed. The complete loss of iron and need for de novo synthesis of hemoglobin likely impede the bone marrow response to hemorrhagic anemia relative to hemolytic anemia (16), suggesting that, if hemolytic rather than blood loss anemia would have been induced, a greater change in reticulocyte number and characteristics might be expected. Accordingly, a Retic of 56.7 × 10^6/liter was reported from a single horse with severe intravascular hemolysis (34).

Horses treated with the low- and high-dose rhEPO had significant changes in RBC and reticulocyte parameters. Although the number of reticulocytes in blood was increased significantly only with the higher dose of rhEPO, changes were present in all reticulocyte parameters and in the CHCM and MCV. Changes early after rhEPO treatment differed little from those after 28 days, except that there was a transient increase in the Hct with the higher dose of rhEPO, which is consistent with a transient effect increasing reticulocyte production. The cellular hemoglobin content of reticulocytes (CHr) and mature RBC (CHCM) most clearly distinguished between the bone marrow response to anemia and EPO. EPO rescues erythroid precursor cells from apoptosis, with colony-forming unit erythroid in bone marrow expressing the highest density of EPO receptors (11). EPO-stimulated colony-forming unit erythroid proliferate and produce reticulocytes after four to six cell divisions and ~7 days (11), which corresponds to the time frame observed in this study of horses. The second administration of rhEPO, at a higher dose and increased frequency in conjunction with iron supplementation, resulted in greater changes in reticulocytes, as would be expected with abundant iron availability (2). In humans, rhEPO increases the relative and absolute concentration of hemoglobin in reticulocytes and the concentration of the soluble transferrin receptor, involved in mediating iron availability for hemoglobin synthesis (20). Effects on these parameters have allowed creation of a model incorporating hemoglobin, reticulocyte, serum EPO, and serum-soluble transferrin receptor concentration to determine the likelihood of rhEPO treatment of human athletes (4, 6, 21). Lack of assays for soluble transferrin receptors and the potential for immune responses against rhEPO to interfere with EPO measurement in serum preclude establishment of such a model in horses. However, the nature of the changes observed in blood reticulocytes suggests that EPO accelerates the release of reticulocytes with increased hemoglobin from the bone marrow of horses. Results of testing for antibodies against rhEPO in equine serum were negative, suggesting that either the horses did not generate antibodies despite two to five treatments with rhEPO or, more likely, the assay lacked sensitivity. Details on the sensitivity and specificity of the ELISA and on the frequency and duration of antibody responses induced in horses treated with rhEPO have not been reported.

Bone marrow aspirates are not readily obtained from horses, and analysis in a hematology analyzer has not previously been reported. The bone marrow of horses, like that of other animals, responds to erythropoietic stress with a relative increase in rubricytes and reticulocytes. Such change was most profound 1 wk after induction of anemia, when a sharp increase in reticulocytes, MCV, MCVr, and CHr was noted. Mean hemoglobin concentration in reticulocytes decreased after anemia but increased steadily after EPO treatment. Neither dose of

<p>| Table 3. Changes in bone marrow RBC parameters after anemia and EPO treatment |</p>
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>Anemia</th>
<th>EPO-L</th>
<th>EPO-H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hct, liter/liter</td>
<td>0.276±0.01</td>
<td>0.244±0.01</td>
<td>0.33±0.02</td>
<td>0.35±0.01</td>
</tr>
<tr>
<td>MCV, fl</td>
<td>47.98±1.25</td>
<td>55.30±1.29</td>
<td>51.47±0.96</td>
<td>53.55±1.03</td>
</tr>
<tr>
<td>Retic, 10^6/l</td>
<td>96.17±26.06</td>
<td>396.20±56.19</td>
<td>74.17±22.88</td>
<td>197.18±38.88</td>
</tr>
<tr>
<td>MCVr, fl</td>
<td>79.22±1.90</td>
<td>92.43±1.90</td>
<td>78.87±1.90</td>
<td>69.71±0.56</td>
</tr>
<tr>
<td>CHr, pg</td>
<td>22.72±0.45</td>
<td>24.75±0.45</td>
<td>22.00±0.45</td>
<td>23.70±0.45</td>
</tr>
<tr>
<td>CHCMr, g/l</td>
<td>294.83±2.74</td>
<td>270.33±2.74</td>
<td>282.00±2.74</td>
<td>284.00±2.74</td>
</tr>
</tbody>
</table>

95% CI for all parameters listed above.
EPO significantly increased bone marrow Retic, MCVr, or CHr, indicating that EPO mainly affected the hemoglobin saturation but not the number or size of reticulocytes. These findings most clearly highlight the different responses of the hematopoietic system to hypoxic stress and supraphysiological concentrations of EPO.

The parameter NCC/Hct (Fig. 3) was calculated to assess the relative cellularity of bone marrow aspirates. All rubricytes, leukocytes, and undifferentiated hematopoietic cells comprise the NCC, which increased sharply in relation to Hct after anemia but not after rhEPO treatment. Although little is known about this calculated parameter in horse bone marrow, it appears likely that the increase in NCC after anemia corresponded to an increase in the rubricyte proportion identified by manual differential counting.

The frequency of rhEPO administered to horses in this study was below that required to enhance athletic performance in humans (4, 32). Daily administration of 50 U/kg to human athletes was required to increase reticulocytes on day 5 and hemoglobin on day 11 (32). To treat the EPO deficiency of chronic renal failure in humans, ~100 U·kg⁻¹·wk⁻¹ of rhEPO were administered (1). Limited pharmacokinetic and efficacy studies of rhEPO in horses indicated that elimination times were similar to those in humans; however, a single dose of 30 U/kg induced no change in hematological parameters in four horses, whereas after three doses of 120 U/kg an increase of 12.5% in the Hct of a single horse was reported (28). The limited number of animals assessed in that study precludes derivation of an optimal dose of rhEPO to increase the RBC mass of horses. From results reported here, it appears that sustained doses of 73 U/kg on alternate days may be necessary to induce durable increases in RBC production. On the other hand, adverse reactions reported in 57% of horses after 14–16 administrations of ~10 U/kg rhEPO (25) were probably due to a vaccine-like immune response against rhEPO and were likely insufficient to significantly increase RBC mass. Thus the ability to detect reticulocytes with unique characteristics in horses with regenerative anemia suggests that their routine assessment is warranted for investigation of equine anemia. Alterations in RBC parameters after 24 h of storage indicate that such assessment should be limited to fresh samples (3). The frequent and high dosages of rhEPO required to increase erythropoiesis, together with the apparent high incidence of adverse reactions, imply that sustained increased aerobic capacity may be difficult to achieve through treatment of horses with rhEPO.

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GRANTS

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REFERENCES


