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# Red blood cell erythropoietin, not plasma erythropoietin, concentrations correlate with changes in hematological indices in horses receiving a single dose of recombinant human erythropoietin by subcutaneous injection

A. K. SINGH
S. GUPTA
A. BARNES
J. M. CARLSON &
J. K. AYERS

*Department of Veterinary Population Medicine, College of Veterinary Medicine, University of Minnesota, St Paul, MN, USA*

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*Ashok K. Singh, Department of Veterinary Population Medicine, College of Veterinary Medicine, University of Minnesota, St Paul, MN 55108, USA. E-mail: singh001@umn.edu*

Erythropoietin (EPO) is a 36-kDa glycoprotein (Tilbrook & Klinken, 1999) that regulates mammalian erythrocyte and hemoglobin (Hb) production (Lacombe & Mayeux, 1998). The amino acid compositions of endogenous horse EPO (eEPO), recombinant human EPO (rhEPO) and darbepoietin (DAR), a synthetic rhEPO analog, are approximately 80% similar and the proteins are nearly identical biochemically and immunologically (Macdougall *et al.*, 1999). Because of structural and functional homology between eEPO and rhEPO or DAR, there is strong perception among horse trainers and owners that rhEPO enhances concentration of the oxygen-carrying red blood cells (RBCs) resulting in an enhancement of the horse's aerobic performance and that rhEPO is undetectable by the current analytical methodologies. This has led to rhEPO doping in horses. Contrary to the belief that rhEPO administration is harmless to the horses, its long-term use results in the development of antibodies against rhEPO that recognize and inactivate endogenous EPO resulting in severe anemia (Piercy *et al.*, 1998). Long-term use of rhEPO can be detected by screening horse plasma for EPO antibodies. The occasional use of rhEPO to enhance performance may not be detected by antibody monitoring and require other sophisticated methods including direct analysis of the peptide in plasma and/or blood hematology indices associated with rhEPO administration. For this purpose, it is important to establish a relationship between blood EPO concentrations and changes in blood hematology indices in horses. Therefore, the goal of this study was to measure plasma EPO and intra-erythrocyte EPO concentrations and blood hematological indices in horses given a single dose of rhEPO by subcutaneous (s.c.) injection. The hypothesis was that the time-course of changes in plasma rhEPO concentrations correlated with the changes in blood hematological indices in rhEPO-administered horses.

Recent studies have shown that rhEPO binds to the surface EPO receptors ($EPO_R$), and initiate a series of cellular processes including RBC maturation and apoptosis (Ghezzi & Brines, 2004). The rhEPO–$EPO_R$ complex is internalized into the $EPO_R$-containing cells where rhEPO is degraded by the lysosomal enzymes (Walrafen *et al.*, 2005; Gross & Lodish, 2006). As RBCs contain $EPO_R$, plasma rhEPO, bound to the $EPO_R$ on RBCs surface, is internalized into the cell. In the absence of lysosomes, rhEPO is not degraded and remain in the RBCs. Thus, the possibility exists that rhEPO may distribute into both plasma and RBCs in rhEPO-administered horses.

Four standardbred female horses with an average body weight of 500 kg were used in these studies. All horses were housed in stalls with periods running free in pasture during the day. The horses were examined by the equine veterinarian and were found healthy. Prior to the rhEPO administration, two sets of blood samples were collected from each horse from juggler vein using heparinized vacutainers and submitted to the ClinPath Laboratory of Veterinary Medical Center for hematological analysis using ADVIA 120 (Bayer Diagnostics, Pittsburgh, PA, USA). The blood hematology profiles were within normal limits. Then, each horse received a single dose (16 000 mU or about 30 mU/kg) of rhEPO by s.c. injection. Blood samples were collected prior to and at different time intervals (0.5, 1, 4, and 12 h and daily for 14 days) after rhEPO administration. A 1-mL aliquot of whole blood was submitted for hematological analysis and the remaining sample was centrifuged and plasma and blood cells were separated. Plasma was stored frozen for EPO analysis. Blood cells were washed twice with phosphate-buffered saline (1:10 w/v) and then hemolyzed with pure water (1:1 v/v). Plasma and the hemolyzed blood cells were analyzed using EPO ELISA kits (M D Biosciences, St Paul, MN, USA).

Distribution of the internalized $EPO_R$ in RBC was studied as follows: RBCs were lysed with pure water and centrifuged at 100 000 ***g*** for 30 min to remove RBC membranes. The supernatant and the membrane fractions were collected, concentrated

 

and loaded onto a 10% polyacrylamide gel. Proteins were separated under 30 mA constant-current mode. The separated proteins were transferred onto a nitrocellulose membrane that was blocked with 5% nonfat milk in tris-buffered saline (TBS) containing 0.5% Tween. Membrane was incubated with $EPO_R$ antibodies raised in rabbits followed by horse radish peroxidase (HRP)-labeled anti-rabbit IgG. Color was developed using appropriate substrate.

For real-time PCR analysis of ζ-globin, blood cells were suspended in 2 mL cold PBS and 5 mL TRI reagent (Sigma Chemicals, St Louis, MO, USA). RNA was extracted according to the manufacturer's protocol. Precipitated RNA was resuspended in 50 μL diethylpyrocarbonate (DEPC) water. A 1-μL aliquot was reverse transcribed with SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) for 60 min at 50 °C in the presence of Oligo-dT. The probes used in this study were:

Zeta (ζ)-Globin: F: 5′-AGCCATCACCATGTCTCTGACCAA-3′
R: 3′-AAACTCACGCTGTCACCTACACCA-5′
P: 5′-FAM- TGAGAGGACCATGGTCGTGTCCATAT-TAMRA-3′
GAPDH: F: 5′-GAAGGTGAAGGTCGGAGTC-3′
R: 5′-GAAGATGGTGATGGGATTTC-3′
P: FAM-CAAGCTTCCCGTTCTCAGCC-TAMRA

The PCR was performed using the ABI Prism SDS in 96-well microtiter plates using a final volume of 25 μL. Amplification was performed as follows: AmparaseUNG activation for 2 min at 50 °C, 10 min template denaturation at 95 °C, 45 cycles of amplification at 95 °C for 15 sec and 60 °C for 1 min. Statistical analysis was performed using SPSS program (SPSS Inc., Chicago, IL, USA). Dunnett's test was used to determine significance between groups at $P < 0.05$.

This study showed that RBC count (Fig. 1a, plot-1), Ht (Fig. 1a, plot-2), and red cell distribution width (RDW) (Fig. 1b, plot-1) values began to increase at 24 h and peaked at approximately 130% of control values at 4 days after rhEPO administration. Hb values began to increase at 2–3 days after RBCs (Fig. 1a, plot 3), and peaked at 4–5 days after rhEPO administration. RBC, Ht, Hb, and RDW values returned to the basal level at about 10 days after rhEPO administration. These observations showed that a single dose of rhEPO significantly increased blood RBC and Hb values, the increase in Hb levels was delayed 2–3 days. This delayed Hb rise may be due to the presence of cells that have not yet synthesized Hb. The rhEPO-induced increase in RDW indicated the presence of anisocytosis characterized by an unusual variation in the size of RBCs in rhEPO-administered horses. Anisocytosis is mainly associated with anemia, the presence of young RBCs, such as polychromatophils, which are larger than mature RBCs and/or the presence of smaller RBCs, such as microcytes or spherocytes (Romero *et al.*, 1999). Thus, blood samples collected immediately after the rhEPO administration may contain immature RBC that may account for anisocytosis in horses. However, the possibility of hematological abnormalities following rhEPO administration cannot be ruled out as a number of hematological abnormalities have been reported in response to EPO abuse in human athletes (Parisotto *et al.*, 2003) and horses (Piercy *et al.*, 1998).

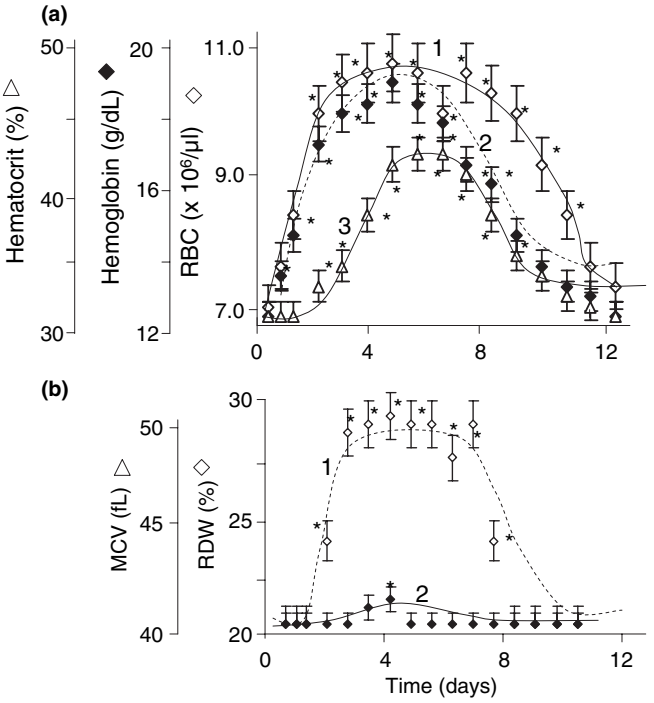


**Fig. 1.** (a) Time course of change in red blood cell (RBC) count (plot 1), hematocrit (Ht) values (plot 2) and hemoglobin (Hb) content (plot 3) in blood samples from recombinant human erythropoietin (rhEPO)-administered horses. (b) Time course of change in red cell distribution width (RDW, plot 1) and mean cell volume (MCV, plot 2) values in blood samples from rhEPO-administered horses. Values are mean ± SD, $n = 4$. *$P < 0.05$, significant when compared with the pre rhEPO-administered values.

Earlier studies have shown that immature RNA-containing RBCs, although produced in horses, are not released during homeostasis or mild anemia (Farnandez & Grindem, 2000). Contrarily, a recent study has shown that anemic horses after rhEPO treatment released reticulocytes into circulation (Cooper *et al.*, 2005). The present study indicated the presence of immature RBCs in blood from rhEPO-administered horses. To further confirm the presence of the RNA-containing immature RBCs in rhEPO-administered horses, levels of mRNA encoding ζ-globin was measured in blood cells. Immature RBCs have been shown to contain (i) a number of functional globin genes that participate in Hb synthesis and (ii) a nonfunctional pseudo-gene maintaining perfect sequence homology with intact ζ-globin gene (Clegg, 1987; Flint *et al.*, 1988). As the cells mature, their Hb content increase but they loose RNA expression and nucleus (Koepke & Koepke, 1986). The present study also showed that mRNA encoding ζ-globin was not present in RBCs collected from horses prior to the rhEPO administration (Fig. 2a). ζ-globin mRNA levels increased gradually and peaked at 6 days after rhEPO administration. ζ-globin mRNA was not detected in RBCs collected at 9 days after rhEPO administration. This suggests that the RNA-containing immature RBCs appear during the early phase of rhEPO administration in horses possibly due to release of immature RBCs from either bone marrow or spleen.

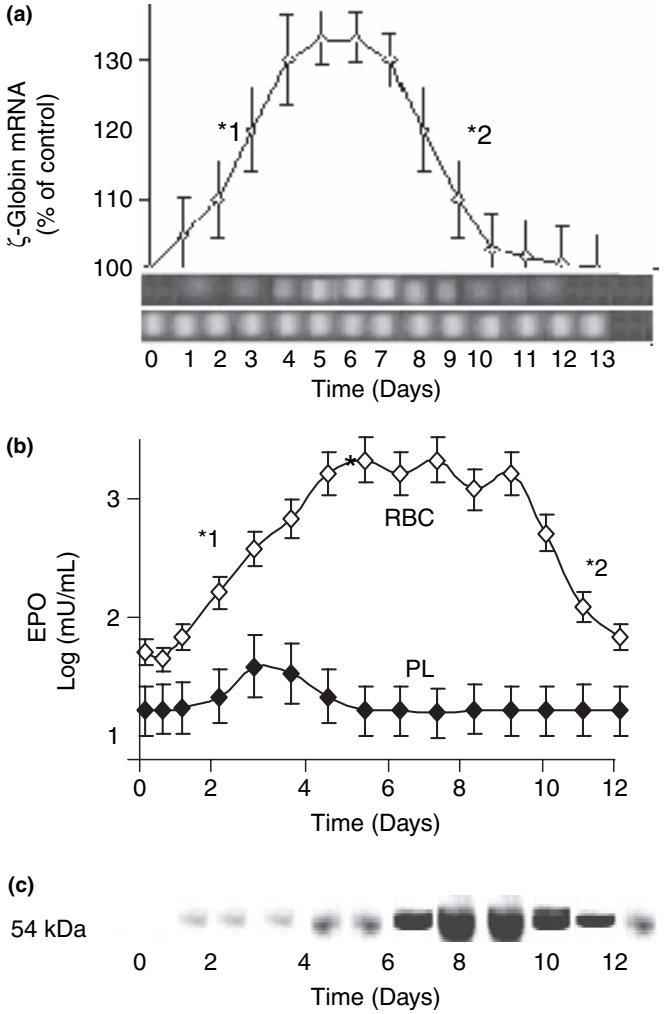


**Fig. 2.** (a) Expression (by RT-PCR) and quantitative analysis (by real-time PCR) of mRNA encoding ζ-globin in red blood cells (RBCs). Values are mean ± SD, $n = 4$. *The mean values significantly higher than the preinjection values ($P < 0.05$). (b) Concentration of rhEPO in plasma and RBC samples from the rhEPO-administered horses. Values are mean ± SD, $n = 4$. *$P < 0.05$, significant when compared with the pre rhEPO injection values. (c) Western blot analysis of internalized $EPO_R$s in RBCs.

It is well established that rhEPO and related peptides bind to a specific membrane receptors, $EPO_R$, and induce a series of cellular processes including cell proliferation, maturation, and apoptosis (Lacombe & Mayeux, 1998). The rhEPO–$EPO_R$ binding (i) stimulates RBC synthesis and release in bone marrow (Mulcahy, 2001) and (ii) regulates maturation and apoptosis in RBCs (Ghezzi & Brines, 2004). Moreover, the persistence of rhEPO in circulation is the key factor that determines the bioactivity of these peptides *in vivo* (Elliott *et al.*, 2004). Thus, we hypothesized that the erythropoietic effects of rhEPO may parallel rhEPO's persistence in plasma. As shown in Fig. 2b, plasma EPO concentrations did not change for the first 12 h and then increased gradually with highest values occurring at 3–4 days after rhEPO administration in horses. EPO values returned to the basal levels within 5–6 days of rhEPO administration. As the biological effects of rhEPO administration lasted for more than 10 days, this study showed that plasma rhEPO concentration may not correlate with rhEPO's biological effects.

A key observation of this study was that, apart from plasma, RBCs also contained EPO and that EPO levels remained elevated in RBCs for up to 13 days after rhEPO administration in horses (Fig. 2b). The time-course of change in RBC EPO concentrations and the bioactivity of rhEPO revealed a positive correlation between the two. Although a mechanism by which rhEPO enter RBCs is not yet known, a recent study has shown that binding of rhEPO to its receptors on the surface of RBCs induces internalization of the rhEPO–$EPO_R$ complex that provides the pathway for degradation of rhEPO and a way to regulate the number of sites that are available for binding on the cell's surface (Walrafen *et al.*, 2005; Gross & Lodish, 2006). Gradually, the number of surface receptors for rhEPO decreases by the accelerated rate of receptor internalization. The present observation that rhEPO administration gradually decreased the RBC-membrane $EPO_R$ but increased the cytoplasmic $EPO_R$ suggests significant internalization of rhEPO in RBCs. As RBCs lack lysosomal enzymes, internalized rhEPO remain intact within RBCs.

The present study, for the first time demonstrates accumulation of EPO in RBCs. This may have significant impact in testing of rhEPO doping in horses. At present, rhEPO doping in horses is commonly detected by directly analyzing the peptide or its antibodies in plasma. This study showed that plasma rhEPO may not be diagnostically relevant because (i) occasional rhEPO administration may not generate antibodies and (ii) rhEPO's bioactivity may last longer than its persistence in plasma. The analysis of rhEPO in RBCs may be a better indicator of rhEPO abuse in horses.

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## REFERENCES

Clegg, J.B. (1987) Can the product of the theta gene be a real globin? *Nature*, **329**, 465–466.

Cooper, C., Sears, W. & Bienzle, D. (2005) Reticulocyte changes after experimental anemia and erythropoietin treatment of horses. *Journal of Applied Physiology*, **99**, 915–921.

Elliott, S., Egrie, J., Browne, J., Lorenzini, T., Busse, L., Rogers, N. & Ponting, I. (2004) Control of rHuEPO biological activity: the role of carbohydrate. *Experimental Hematology*, **32**, 1146–1155.

Farnandez, F.R. & Grindem, C.B. (2000) Reticulocyte response. In *Schalm's Veterinary Hematology*, Eds Feldman, B.F., Zinkl, J.G. & Jain, N.C., pp. 110–116. Lipincott, Philadelphia, PA.

Flint, J., Taylor, A.M. & Clegg, J.B. (1988) Structure and evolution of the horse zeta globin locus. *Journal of Molecular Biology*, **199**, 427–437.

Ghezzi, P. & Brines, M. (2004) Erythropoietin as an antiapoptotic, tissue-protective cytokine. *Cell Death & Differentiation*, **11,** S37–S44.

Gross, A.W. & Lodish, H.F. (2006) Cellular trafficking and degradation of erythropoietin and novel erythropoiesis stimulating protein (NESP). *Journal of Biological Chemistry*, **281,** 2024–2032.

Koepke, J.F. & Koepke, J.A. (1986) Reticulocytes. *Clinical & Laboratory Haematology*, **8,** 169–179.

Lacombe, C. & Mayeux, P. (1998) Biology of erythropoietin. *Haematologica*, **83,** 724–732.

Macdougall, I.C., Gray, S.J., Elston, O., Breen, C., Jenkins, B., Browne, J. & Egrie, J. (1999) Pharmacokinetics of novel erythropoiesis stimulating protein compared with epoetin alfa in dialysis patients. *Journal of the American Society of Nephrology*, **10,** 2392–2395.

Mulcahy, L. (2001) The erythropoietin receptor. *Seminars in Oncology*, **28** (Suppl. 8), 19–23.

Parisotto, R., Ashenden, M.J., Gore, C.J., Sharpe, K., Hopkins, W. & Hahn, A.G. (2003) The effect of common hematologic abnormalities on the ability of blood models to detect erythropoietin abuse by athletes. *Haematologica*, **88,** 931–940.

Piercy, R.J., Swardson, C.J. & Hinchcliff, K.W. (1998) Erythroid hypoplasia and anemia following administration of recombinant human erythropoietin to two horses. *Journal of the American Veterinary Medical Association*, **212,** 244–247.

Romero, A.J., Carbia, C.D., Ceballo, M.F. & Diaz, N.B. (1999) Red cell distribution width (RDW): its use in the characterization of microcytic and hypochromic anemias. *Medicina*, **59,** 17–22.

Tilbrook, P.A. & Klinken, S.P. (1999) Erythropoietin and erythropoietin receptor. *Growth Factors*, **17,** 25–35.

Walrafen, P., Verdier, F., Kadri, Z., Chretien, S., Lacombe, C. & Mayeux, P. (2005) Both proteasomes and lysosomes degrade the activated erythropoietin receptor. *Blood*, **105,** 600–608.