Biological Activity of Recombinant Human Erythropoietin (EPO) *In Vivo* and *In Vitro*

Park, Jong-Ju[1], Hyen-Gi Lee[1,2], In-Suk Nam, Hee-Ja Park, Min-Su Kim, Yun-Hi Chung, Purevjargal Naidansuren, Hye-Young Kang, Poong-Yun Lee[2], Jin-Gi Park[2], Hwan-Hoo Seong[2], Won-Kyong Chang[2], Myung-Hwa Kang[3], Yong-Soo Park[4], Seong-Soo Hwang[5], Sue-Yun Hwang[5], Jong-Taek Yoon[5] and Kwan-Sik Min[5†]

Animal Biotechnolgy, The Graduate School of Bio. & Information Technology, Hankyong National University

ABSTRACT

The hematopoietic growth factor erythropoietin (EPO) is required for the maintenance, proliferation, and differentiation of the stem cells that produce erythrocytes. To analyse the biological activity of the recombinant human EPO (rec-hEPO), we have cloned the EPO cDNA and genomic DNA and produced rec-hEPO in the CHO cell lines. The growth and differentiation of EPO-dependent human leukemic cell line (F36E) were used to measure cytokine dependency and *in vitro* bioactivity of rec-hEPO. MTT assay values were increased by survival of F36E cells at 24 h or 72 h. The hematocrit and RBC values were increased by subcutaneous injection of 20 IU (in mice) and 100 IU (in rats) rec-hEPO. Hematocrit values remarkably increased at 13.2% (in mice) and 12.2% (in rats).

The pharmacokinetic behavior with injection of 6 IU of rec-hEPO remained detectable after 24 h in all mice tested. The highest peat appeared at 2 h after injection. The long half-life of rec-hEPO is likely to confer clinical advantages by allowing less frequent dosing in patients treated for anemia. These data demonstratethat rec-hEPO produced in this study has a potent activity *in vivo* and *in vitro*. The results also suggest that biological activity of rec-hEPO could be remarkably enhanced by genetic engineering that affects the potential activity, including mutants with added oligosaccharide chain and designed to produce EPO-EPO fusion protein.

(Key words : Erythropoietin, Recombinant, Biological activity, Pharmacokinetic behavior)

INTRODUCTION

Human erythropoietin (hEPO) is a glycoprotein produced from a 193-amino acid gene product after a leader sequence containing 27 amino acids is cleaved. A carboxy-terminal arginine is lost from this 166-amino acid residue during passage into the circulation leaving a circulating hormone with 165 amino acids. The molecular mass of the EPO peptide is 18 kDa (Lai et al., 1986). However, the glycoprotein entity as a whole is 30 kDa (Davis et al., 1987). EPO is heavily glycosylated with a carbohydrate moiety of approximately 40%. Three N-linked (Asn-24, 38 and 83) and one O-linked (Ser-126) oligosaccharide side chains are contained in the molecule (Broudy et al., 1987; Egrie et al., 1986; Sasaki et al., 1987; Takeuchi et al., 1989). Human EPO contains 4 cysteine residues that form 2 disulfide bridges between cysteine 7 and 161 and between cysteine 29 and 33 (Lai et al., 1986).

Early studies indicate that the liver is the primary site of production of EPO in the fetus, and there is a gradual shift from the liver to the kidney shortly after birth (Sasaki et al., 1987; Zanjiani et al., 1981). The switch from liver to the kidney is gradually initiated at 120~140 days of gestation and is completed around 40 days after birth (Zanjiani et al., 1981). However, the signalsgoverning this change are poor understood. In the liver, an oxygen gradient is established as oxygen-rich blood from the portal triads becomes depleted of oxygen as it flows towards the central vein (Benjamin and Franklin, 1999).

* This study was supported by the BIO-GREEN 21 PROGRAM.

[1] These authors have contributed equally to this work

[2] Animal Biotechnology Division, NLRI, RDA.

[3] Department of Food and Nutrition, Hoseo University.

[4] Kyongbuk Livestock Research Institute.

[5] Institute of Genetic Engineering, Hankyong National University.

† Corresponding author : Dr. Kwan-Sik Min, The Graduate School of Bio. & Information Technology, Hankyong National University, Ansung, Gyeonggi 456-749, Korea, E-mail: ksmin@hknu.ac.kr

We previously prepared recombinant hEPO in CHO-K1 cells, and found that the deglycosylated sites varied the expression of these recombinant derivatives (Lee et al., 2003). In the presented study, we cloned hEPO cDNA and genome DNA and compared the expression quantity. The biological activity of recombinant hEPO was determined by the measuring MTT assay *in vitro* and hematocrit value and pharmacokinetics *in vivo*.

MATERIALS AND METHODS

Materials

The expression vector pcDNA3 was purchased from Invitrogen Life Technologies (CA, USA). CHO-K1 cells and Human leukemic cell line (F-36E) were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). Ham's F-12, CHO-S-SFM II, G418 and Lipofectamine were from Gibco BRL (MD, USA). Fetal bovine serum was from Hyclone Laboratories (Utah, USA). Endonucleases were purchased from Takara Korea Biomedical Inc. (Seoul, Korea) and Boehringer Mannheim (MA, USA). The PCR primers synthesized by CoreBio System (Seoul, Korea). The QIAprep-spin plasmid kit was from QIAGEN Inc. (Hilden, Germany). EPO Elisa kit was from Medac (Germany) and R&D systems Inc. (MN, USA). All the other reagents used were from Wako Pure Chemicals (Osaka, Japan).

Construction of EPO Transfer Vector

The primers [sense: 5'-TAg AAT TCA CCA Tgg ggg TgC Acg AAT gTC CT-3' and antisense: 5'-ACT CgA gTT CAT CTg TCC CCT gTC CTg CA-3'] were designed from the nucleotide sequence of the EPO cDNA reported previously. PCR template was used with human liver cDNA library from Takara. And genome EPO DNA was amplified from human placental genome DNA using sense primer [5'-ggT ACC Atg ggg gTg CAC ggT gAg TAC TCg-3'] and the same antisence of EPO cDNA. PCR fragments were ligated into PCR2.1 Vector and sequenced completely to confirm the Kozak site and PCR errors. After digested with *Eco* RI - *Xho* I (cDNA) and *Kpn* I *Xho* I (gDNA), the fragments were inserted into the same sites of pcDNA3.

Cell Culture and Functional Expression

The expression vector (pcDNA3) was transfected into CHO-K1 cells by the liposome formulation (Lipofectamine) transfection method according to the supplier's instruction. rec-hEPO protein transfected by transient was collected the supernatants at 72 h after transfection. Stable cell transfectants were selected by incubation in growth medium [Ham's F12 media containing penicillin (50 U/ml), streptomycin (50 mg/ml), glutamine (2 mM) and 10% FCS] supplemented with G418 (800 ug/ml) for 2 weeks posttransfection according to the method reported previously (Min et al., 2004). After incubation of selected stable cells (1×10^6) in 20 ml CHO-S-SFM-II at 37℃ for 48 h, the culture media were collected and centrifuged at 100,000×g, 4℃ for 60 min to remove the cell debris. The amount of recombinant wild type hPEO was quantified using ELISA method according to the supplier's protocol (R&D systems Inc. MN, USA).

Assay for Cell Proliferation (*In Vitro* Biological Activity)

Short-term cell proliferation was examined by a colorimetric assay. EPO-dependent human leukemic cell lines (F-36E) were incubated at a density of 1×10^4 cells/100 ul in 96-well plates in growth medium [DMEMcontaining penicillin (50 U/ml), streptomycin (50 mg/ml) and 10% FCS] in the presence of 0-5 IU/ ml of rec-hEPO for dose- response plots. Next, MTT reagent was added at a final concentration to 0.5 mg/ml. Following a 4-hour incubation at 37℃, the isopropylalcohol with 0.04 N HCl was added to each well. Within an hour, the 96-well plates were measured at 630 nm. MTT was added after 24 to 72 hours of incubation.

In Vivo Biological Activity

Two groups of mice (ICR) and rats (WsRc-Wa/Wa: anemia, mast cell depletion and c-kit abnormality) were injected subcutaneously with 20 IU (mice) and 100IU (rat) of rec-hEPO on days 0, 2 and 4. Blood was withdrawn for hematocrit determination. Hematocrit values measured at 8 (mice) and 10 (rats) days after the first injection.

In Vivo Pharmacokinetics

Two groups of ICR-mice (7 weeks old) were used. Before the administration of rec-hEPO, 70~80 ul of blood were collected from the eyes into heparinized microhematocrit tubes. The tubes were centrifuged, and plasma was collected and frozen at -80℃. Each animal received 6 IU of rec-hEPO by intravenous injection, and blood samples were obtained at 2 h, 4h, 8 h and 24 h thereafter. All intravenous injections were performed by skilled technical personnel and monitored independently.

RESULTS

PCR Amplification and Cloning of EPO cDNA and Genome DNA

Using the cDNA prepared from liver cDNA library and genome DNA from human placenta, PCR was amplified the primers designed from the sequences published previously (Fig. 1). The cDNA fragment with 577 bp predicted for EPO cDNA was amplified. A genome DNA band of about 2 kb for the EPO genome DNA was amplified using a set primers. Each fragment was subcloned into PCR2.1 cloning vector and sequenced. Analysis of the nucleotide and

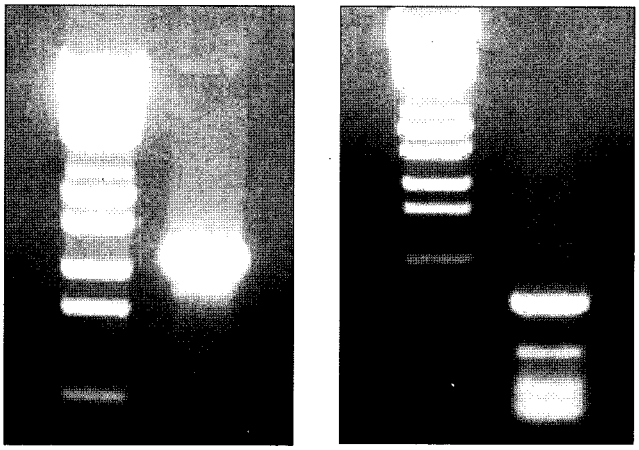

Fig. 1. PCR amplification of human EPO cDNA and genome DNA. The PCR products predicted with each primer were analyzed by 1.0% agarose electrophoresis. The major fragments obtained, which comprised 2.0 kb (gDNA: left) and 577 bp (cDNA: right), were amplified.

genome sequence of the EPO was identical to the previous data reported.

Production of Transient and Stably Expressing EPO

EPO cDNA and genome DNA vectors were transfected into CHO-K1 cells. Transient expression was collected the supernatants at 72 h after transfection. Between six and eight clones of stably transfected pools were selected for G-418. After incubation of selected stable cells (1×10^6) in 20 ml CHO-S-SFM-II at 37℃ for 48 h, the culture media were collected and centrifuged at 15,000 rpm, 4℃ for 60 min to remove the cell debris. The amount of rec-hEPO was quantified using ELISA (Fig. 2). There is no different between EPO cDNA and genome DNA in the expression quantity (Fig. 2).

Assay for Cell Proliferation (*In Vitro* Biological Activity)

EPO is the most potent cytokine for the F-36E cell lines. The growth and proliferation of the F-36E cells by EPO concentrations were shown in Fig. 3. The proliferation of the cells was increased by the EPO concentration in 24 h and 72 h. Specially, the maximal stimulation by EPO was almost equal to this cell lines in short-term assay.

In Vivo Biological Activity

Two groups of mice were injected subcutaneously with 20 IU of rec-hEPO on days 0, 2 and 4. Hematocrit values measured 8 days after the first injection. Mean hematocrit values were remarkably increased from 51.1 % to 64.3 %. The values of negative control groups were not raised. Average values were 49.05 % and 50.12 % pre- and post injection, respectively (Fig. 4A and B). And RBC values were measured at the same time. Mean RBC values were slightly increased from 9.87 to 11.85 (Fig. 4C and D).

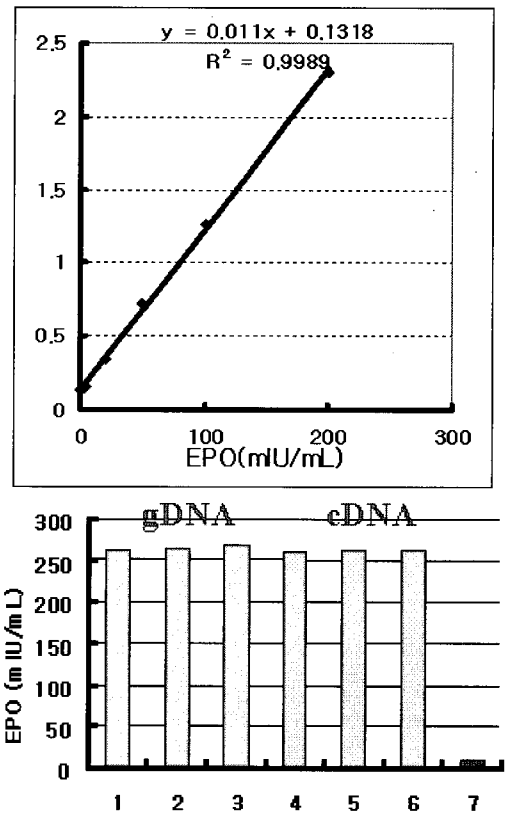

Fig. 2. Transient expression of EPO cDNA and genome DNA. ELISA result of standard curve was shown (left). The transient expression of EPO cDNA and genome DNA was shown (right). The amount of expression is similar to each other. Experiments were performed in triplicate (1~3: cDNA; 4~6: genome DNA; 7: negative control).

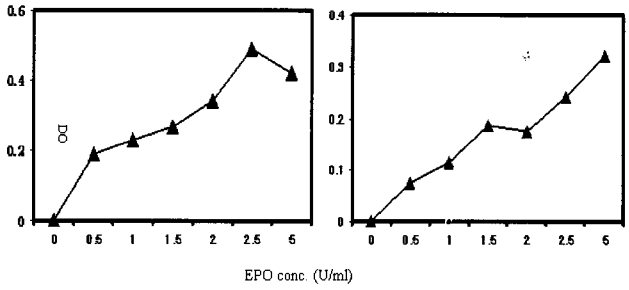

Fig. 3. Responsiveness of F-36E cell lines for rec-hEPO (left: 24 h; right: 72 h). The results are shown as a mean triplicate data.

To compare the relative in mice and rat, rats (WsRc-Wa/Wa: anemia, mast cell depletion and c-kit abnormality) wereinjected subcutaneously with 100 IU of rec-hEPO. Mean hematocrit values were significantly raised from 40.1 % to 52.3 % (Fig. 5A and B). RBC values were also slightly increased from 5.22 % to 6.85 % (Fig. 5C and D). The hematocrit values of rat were below about 10 % than that of mice. This reason is that rat used in this study was anemic experimental animal of mast cell depletion and c-kit abnormality.

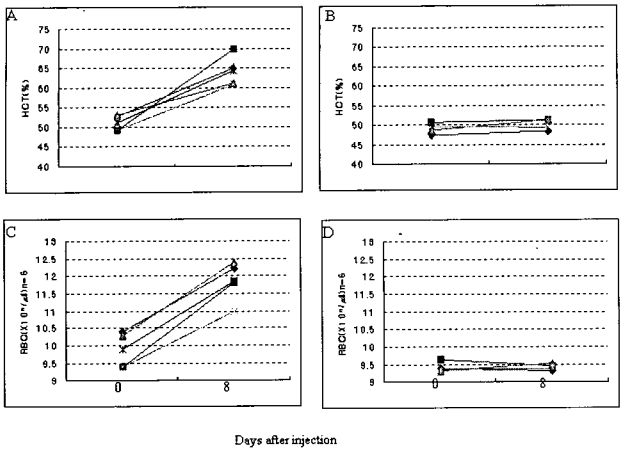

Fig. 4. EPO induces high hematocrit and RBC increase in mice. Two groups of ICR mice were injected subcutaneously with 20 IU of rec-EPO on day 0, 2 and 4. Hematocrit (A and B) and RBC (C and D) were determined pretreatment (0) and 8 days after injection. Values are given for each mouse.

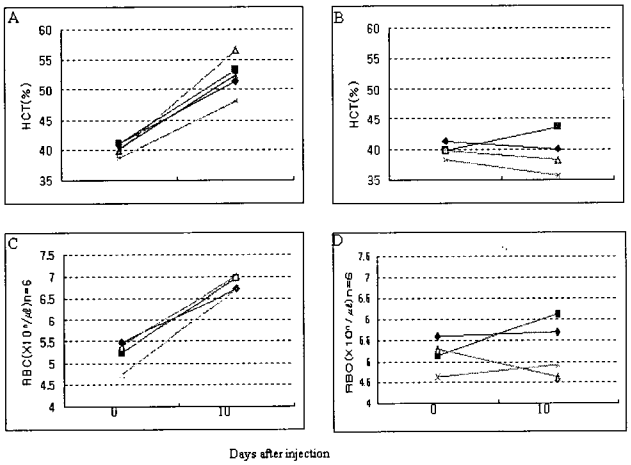

Fig. 5. EPO induces high hematocrit and RBC increase in rats. Two groups of WsRc-Wa/Wa rats were injected subcutaneously with 100 IU of rec-EPO on day 0, 2 and 4. Hematocrit (A and B) and RBC (C and D) were determined pretreatment (0) and 10 days after injection. Values are given for each rat.

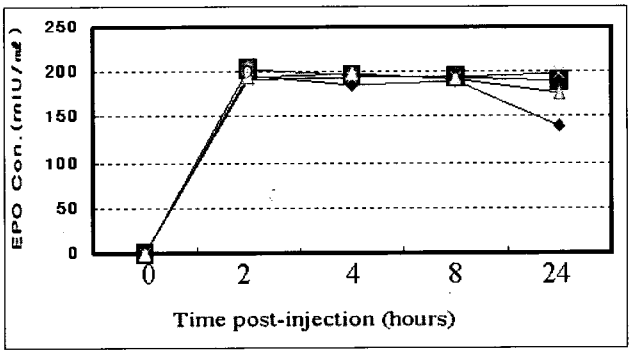

Fig. 6. Pharmacokinetics of rec-EPO in mice. Groups of four mice were injected intravenously with 6 IU of EPO. Each symbol represents one animal. EPO production is given as the maximum level observed 2 h after injection.

Pharmacokinetic Analyses

Two groups of ICR-mice were used in pharmacokinetic analyses (Fig. 6). Each animal received 6 IU of rec-hEPO by intravenous injection, and blood samples were obtained at 2 h, 4h, 8 h and 24 h thereafter. At specified times, blood samples were obtained, and the plasma concentration of EPO was determined by ELISA. All four animals injected were the highest peak at 2 h after injection. EPO levels remained high for 24 h after injection and detectable after 24 h in all four animals. One of the four animals was slightly decreased after 24 h.

DISCUSSION

In the present study, we have shown that rec-hEPO produced into the CHO cell lines has an enhanced biological activity *in vitro* and *in vivo*. The results of these biological activity show that rec-hEPO could be remarkably enhance by the genetic engineering. A major advance in clinical medicine over the past several years has been the use of recombinant human EPO in the treatment of patients with anemia associated with end-stage renal disease that are being maintained on dialysis.

We carried out the ELISA analysis of the culture media of the EPO cDNA and genome DNA to compare the expression quantity. There was no different in the expression quantity. Previously, we produced glycoprotein hormones (equine chorionic gonadotropin, follicle stimulating hormone and luteinizing hormone) and showed that this glycoproteine hormone can be increase biological activity (Min et al., 2003, 2004; Saneyoshi et al., 2001). The EPO-dependent cell lines (F36E) have been established in human leukemia cells (Chiba et al., 1991). We also assayed the growth and proliferation of F36E cell by the dose-responsiveness of rec-hEPO. The dose-dependent curve of rec-hEPO is increased by the rec-hEPO addition during 24 h and 72 h of incubation. The hematocrit value after injected on days 0, 2, and 4 in normal mice (ICR) with EPO 20 IU was highly increased at 13.2%. In anemic rats (WsRc-Wa/Wa) after injected at concentrations of 100 IU, the value of hematocrit also remarkably increased at 12.2%. The pharmacokinetic behavior of rec-hEPO remained detectable after 24 h in all four animals. These data suggest that rec-hEPO has a potent activity *in vivo*. Thus, rec-hEPO has an enhanced biological activity. It suggests that rec-hEPO produced in this study has enable to therapy the patients with anemia.

Recently, the dimeric EPO molecule has been proven by several experiments on the highly erythropoietic activity *in vitro* and *in vivo*(Dalle et al., 2001; Sykowski et al., 1999; Qiu et al., 1998). Darbepoetin alfa, a hyperglycosylated rec-hEPO ananlogue with two extra carbohydrate chain, has approximately 13-fold to 14-fold more potent than rec-

hEPO. (Egrie and Browne. 2001 Egrie et al., 2003). Macdougall et al., (1999) reported a similar result that NESP (novel erythropoietin stimulating protein), a hyperglycosylated analogue of rec-hEPO (Epoetin), has an increased terminal half-life in animal models.

Finally, these are the result of the producing combination of the information gained from both genetic and biochemical approaches. Dimeric-EPO can be permit development of potent new analogues that stimulate the hematocrit value. The constructs of dimeric molecule will also be useful in the study of mutants that affect monomeric association and/or secretion. Dimeric analog can also be constructed to include additional specific bioactive generating potentially efficacious. Recombinant EPOs including the mutants, thatlack oligosaccharides, will be useful tools for analyzing the structure-function relationships.

ACKNOWLEDGMENTS

I wish to thank the Japanese Cancer Research Resources Bank for donating the CHO-K1 cells.

REFERENCES

1. Benjamin LE, Franklin B (1999): Regulation of the erythropoietin gene. Blood 94:1864-1877.
2. Broudy VC, Tait JF, Powell JS (1988): Recombinant human erythropoietin: Purification and analysis of carbohydrate linkage. Arch Biochem Biophys 265:329-336.
3. Dalle B, Henri A, Rouyer-Fessard P, Bettan M, Scherman D, Beuzard Y, Payen E (2001): Dimeric erythropoietin fusion protein with enhanced erythropoietin activity *in vitro* and *in vivo*. Blood 97:3776-3782.
4. Davis JM, Arkawa T, Strickland TW, Yphantis DA (1987): Characterization of recombinant human erythropoietin produced in Chinese hamster cells. Biochemistry 26: 2633-2638.
5. Egrie JC, Browne JK (2001): Development and characterization of novel erythropoiesis stimulating protein (NESP). Nephrol Dial Transplant 3:3-13.
6. Egrie JC, Strickand TW, Lane J, Aoki K, Cohen AM, Smalling R, Trail G, Lin FK, Browne JK, Hines DK (1986): Characterization and biological effects of recombinant human erythropoietin. Immunology 172:213-244.
7. Egrie JC, Dwyer E, Browne JK, Hitz A, Lykos MA (2003): Darbepoetin alfa has a longer circulating half-life and greater *in vivo* potency than recombinant human erythropoietin. Exp Hematol 31:290-299.
8. Lai PH, Everett R, Wang FF, Arakawa T, Goldwasseer E (1986): Structural characterization of human erythropoietin. J Biol Chem 261:3116-3121.
9. Lee HG, Lee PY, Lee YK, Kim SJ, Chung HK, Seo MK, Park JK, Min KS, Chang WK (2003): Effects of changes in glycosylation sites on secretion of recombinant erythropoietin in cultured CHO cells. Korean J Animal Reprod 27:299-307.
10. Macdougall IC, Gray SJ, Elston O, Breen C, Jenkins B, Browne J, Egrie J (1999): Pharmacokinetics of novel erythropoiesis stimulating protein compared with epoetin alfa in dialysis patients. J Am Soc Mephol 10:2392-2395.
11. Min KS, Hiyama T, Seong HH, Hattori N, Tanaka S, Shiota K (2004): Biological activities of tethered chorionic gonadotropin (eCG) and its deglycosylated mutants. J Reprod Dev 50:297-304.
12. Min KS, Kang MH, YoonJT, Jin HJ, Seong HH, Chang YM, Chung HJ, Oh SJ, Yun SG, Chang WK (2003): Production of biological active single chain bovine LH and FSH. Asian-Aust J Anim Sci 16:498-503.
13. Qiu H, Belanger A, Yoon HWP, Bunn HF (1998): Homodimerization restores biological activity to an inactive erythropoietin mutant. J Biol Chem 273:11173-11176.
14. Saneyoshi T, Min KS, Ma XJ, Nambo Y, Hiyama T, Tanaka S, Shiota K (2001): Equine follicle-stimulating hormone: Molecular cloning of b-subunit and biological role of the asparagine-linked oligosaccharide at asparagine56 of a-subunit. Biology of Reproduction 65: 1686-1690.
15. Sasaki H, Bothner B, Dell A, Fukuda M (1987): Carbohydrate structure of erythropoietin expressed in Chinese hamster ovary cells by a human erythropoietin cDNA. J Biol Chem 262:12059-12076.
16. Sytkowar AJ, Lunn ED, Risinger MA, Davis KL (1999): An erythropoietin fusion protein comprised of identical repeating domains exhibits enhanced biological properties. J Biol Chem 27:24773-24778.
17. Takeuchi M, Inoue N, Strickland TW, Kobata M, Wada M, Wada M, Shimizu R, Hoshi S, Kozutsumi H, Takasaki S, Kobata A (1989): Relationship between sugar chain strusture and biological activity of recombinant human erythropoietin produced in Chinese hamster ovary cells. Proc Natl Acad Sci USA 86:7819-7822.
18. Zanijani ED, Ascensao JL, McGlave PB, Banisadre M, Ash RC (1981): Studies on the liver to kidney switch of erythropoietin production. J Clin Invest Med 89:640-644.

(Received: 10 March 2005 / Accepted: 25 March 2005)