

Reprint from

RECENT ADVANCES
IN DOPING ANALYSIS
(5)

W. Schänzer
H. Geyer
A. Gotzmann
U. Mareck-Engelke
(Editors)

Sport und Buch Strauß, Köln, 1998

S.M YANG, X.B WANG, Z.L FANG, SH. WANG, Y. QIN, M.Q YI,
H. GAO, B.H XU, L. ZHOU, F.X ZENG, J.J GUO, ZH. Y YANG,
J. DENG, M.H XI, Z.Y YANG, CH.J ZHANG:

Observation on Hematology and Biochemistry of Six Chinese Following rhEPO
Administration

In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in
doping analysis (5). Sport und Buch Strauß, Köln, (1998) 215-221

Observation on Hematology and Biochemistry of Six Chinese following rhEPO Administration

*S.M Yang, X.B Wang, Z.L Fang, Sh Wang, Y. Qin, M.Q Yi, H.Gab, B.H Xu,
L.L Zhou, F.X Zeng, J.J Guo, Zh. Y Yang, J. Deng, M.H Xie, Z.Y Yang, Ch.J Zhang
Doping Control Centre of China, Beijing*

ABSTRACT

Six male volunteers (age 19 to 23) were subcutaneously administered by rhEPO with a dosage of 30 IU/kg bwt, three times a week for 4 weeks. The observation was made on 6 hematological (RBC, HGB, HCT, MCV, MCH and MCHC) and 2 biochemical indices (EPO, TfR) following the administration. Compared with 4 individuals in control group (age 19 to 23) RBC, HGB and HCT were elevated by the injection (4.65 ± 0.33 vs 5.07 ± 0.38 t/l, 134.50 ± 8.2 vs 150.10 ± 10.1 g/l, 41.76 ± 1.93 % vs 46.42 ± 2.83 %; $P < 0.05$). The serum concentrations of TfR and EPO were found significantly increased following the multiple injections of rhEPO (3.42 ± 0.60 ug/ml vs 4.56 ± 0.88 ug/ml, 9.96 ± 8.30 mIU/ml vs 19.94 ± 10.50 mIU/ml; $P < 0.05$).

key words

recombinant erythropoietin; ELISA; Chinese; hematology; transferrin soluble receptor

INTRODUCTION

Erythropoietin (EPO) is the principle hormone regulating the mammalian erythrocyte differentiation . It is produced by the cells adjacent to the proximal renal tubules in response to signals from the renal oxygen-sensing device. Since DNA-recombinant products (rhEPO) is available on the market it has been used as replacement agent in the patients with impaired production of EPO. It was also demonstrated that EPO increases the erythrocyte production and dramatically improve the sense of well-being and quality of life in the patients with chronic renal disease ⁽¹⁾

In the study involving healthy individuals it has been reported that rhEPO administration will increase the haemoglobin concentration with a related increase in VO_{2max} and improve the performance during a standard maximal treadmill run ^(2,3) .

Because of the obviousness as a threat to the fair competition in sports and the health of athletes, the Medical Subcommission of International Olympic Committee has put rhEPO into the list of banned drugs in 1990 in spite of no effective method used in doping test. Since then, many work has been proceeded for the methodological development. In these studies, hematological observation and immunoassay in blood did take an important part. Hematological indices (such as HCT, RBC, HGB) and the blood levels of EPO and TfR exhibit a significant elevation after the administration of rhEPO to normal individuals. These elevation would be used in the screening for the EPO-suspect case.

Unfortunately, the dope analyst have to be faced with a challenge, that is, since the hypoxia can dramatically stimulate secretion of EPO altitude training would play a role in the elevation of blood EPO and in the consequent changes of hematology and biochemistry⁽⁴⁾ .

To discriminate the exogenous injection from the endogenous EPO an important proceeding has been successfully made with electrophoresis isolation based on the different median charge possessed by the endogenous EPO and rhEPO⁽⁵⁾. In this work an index in terms of mAMU (albumin mobility units) was presented, which is the electrophoretic mobility of the activity in relation to that of human serum albumin. If the mAMU gotten in serum analysis by electrophoresis is smaller than 670 it would be an indication of rhEPO's existance . More conveniently, this method could be used in urine analysis although it should be further evaluated.

As an initial study to demonstrate the feasibility and validity of immunoassay in EPO test we conducted this observation. 10 volunteers participated in the experiment, 6 hematological and 2 biochemical indices were observed.

METHODS

Subjects

10 male volunteers (aged 19 to 23yr, mean 21yr) were random divided into two groups: control group N=4 and rhEPO-treated group N=6. For clinic diagnosis they are healthy and in a moderate training and labour work. The volunteers gave their written consent to participate. The study was approved and sponsored by Anti-Doping Commission of China.

rhEPO treatment and sampling

rhEPO- β , (Recomon 2000, Boehringer Mannheim GmbH, Germany) was subcutaneously administered to the 6 volunteers in rhEPO-treated group, 30 IU/kg bwt, three times a week for 4 weeks. Ferric fumarate (200mgx3/day) and folic acid (5mgx3/day) were orally administered to both the control group and the rhEPO-treated group.

10ml of blood was taken at 8:00 to 10:00 am, added with heparin the blood was centrifuged, the serum and plasma was kept at -70°C.

Sampling was begun from day 1 before injection and continued to day 3, day 9 and day 11 after the first injection; from day 15, every two days till day 29 after the first injection. The last two sampling was scheduled on day 34 and day 40 after the first injection. Totally 140 specimens were gotten.

Morning urine was collected in a volume of 100ml for the urinary assay of fibrin and fibrinogen degradation products (the result was not included in this paper).

Hematological observation

6 indices including haematocrit (HCT), red blood cell count (RBC) and concentration of haemoglobin(HGB), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were determined using Automatic Hemology Analyzer (Cell-Dyn 1400, Abbott Co. U.S.A). Statistics was made to get average, standard deviation and, Student's T-test (One-tailed) adopted for checking the difference.

Biochemical measurement

Serum EPO and serum TfR were measured using ELISA (enzyme linked immunosorbent assay) (R&D Systems, Inc. Minneapolis, U.S.A.) for the all 140 specimens. According to the manufactures' specification, the raw data was gotten on a Microplate Autoreader (EL-311, Bio-Tek Instruments , Winooski, VT, U.S.A).

RESULT

Hematological indices

As shown in Tab 1, out of the 6 indices, RBC, HGB and HCT of the rhEPO-treated group were increased by rhEPO injection compared with the control group ($P < 0.05$), although the averages of the levels of both the control group and the rhEPO-treated group are still within the normal range (RBC: 4.00-5.50 $\times 10^{12}/l$, HGB: 120-160 g/l, HCT: 40-51%, referred from a clinic statistics in An Zhen Hospital , Anding Road 11, Beijing). The levels of MCV, MCH and MCHC were not shown with a significant difference by this study.

Tab 1 Summary of Hematological Measurements

index	unit	control group N=4	rhEPO-treated group N=6
RBC	$10^{12}/l$	4.65 ± 0.33	$5.07 \pm 0.38^*$
HGB	g/l	134.50 ± 8.2	$150.10 \pm 10.1^*$
HCT	%	41.76 ± 1.93	$46.42 \pm 2.83^*$
MCV	fl	90.15 ± 4.22	$91.86 \pm 4.45^{**}$
MCH	pg	29.14 ± 1.42	$29.90 \pm 1.46^{**}$
MCHC	g/l	323.65 ± 6.64	$325.45 \pm 5.08^{**}$

* $P < 0.05$ **n.s

Biochemical measurements

As shown in Tab 2, the assays indicates that the serum level of EPO and TfR could be apparently elevated by rhEPO injection. The level of the control group is 9.96 ± 8.30 mIU/ml for EPO and 3.42 ± 0.60 ug/ml for TfR. The former can hit the normal range

provided by the manufacture's specification (EPO: 3.3-16.6 mIU/ml, from 123 normal individual)⁽⁶⁾, the latter is slightly higher than the upper limit of normal range (0.85-3.05 ug/ml, from 1000 healthy individuals)⁽⁷⁾. However, the levels of EPO and TfR in the rhEPO-treated group give out a significant difference compared with the respective control group ($P < 0.05$).

Tab 2 Summary of Biochemical Assays

index	unit	sample	control N=4	rhEPO-treated N=6
EPO	mIU/ml	serum	9.96±8.30	19.94±10.51*
TfR	ug/ml	serum	3.42±0.60	4.56±0.88*

* $P < 0.05$

DISCUSSION

1. Within the rhEPO-treated group a longitudinal change of the individuals could be seen in the plot of the concentration of EPO vs time in day (Fig.1, Fig.2) with a big promotion on the third day after the first injection but, without an apparent drawing back after the injection stopped (sampling points of 4 days and 10 days after the *last* injection). This is baffling since the detectable duration of EPO reported in blood is only 24 h^(8,9). The reason might be the inaccuracy of the assay or some other things left unknown.

The plot of TfR concentration vs time exhibits a smooth increase as the volunteers multiply injected by rhEPO. A big promotion appeared on the 9th day after the first injection and an obvious drawing back could be seen after the injection stopped (Fig.3, Fig.4). As reported by many researches the promotion of TfR in blood after rhEPO injection will appear with a hysteresis relatively to the secretion of reticulocyte and its getting into the circulation⁽¹⁰⁾ and therefore, a prolonged drawing back should be seen on the plotting. While in our study we failed to demonstrate the hysteresis after the injection stopped, for which we considered it appears after the EPO's drawing back. Obviously, it is difficult to show the hysteresis with a large interval of sampling and in such small population and, a study special for the longitudinal investigation should be performed in a large-enough population before TfR could be actually used.

2. The measurement of the hematological changes provides a supplementary index for the rhEPO's possible existence. Together with the analysis of EPO and TfR in serum and/or in plasma a screening method could be formed for EPO dope, which might be economic and acceptable. Although some hematological indices (MCV, MCH and MCHC) did not exhibit a promoted level in our experiment (but did in some other work) HCT, RBC and HGB might be enough as a supplement in the screening, which are reported by many experts as a confirmable utility in their study.

A problem probably encountered in the exploitation of these indices is that in our study, the subjects in rhEPO-treated group are not demonstrated by any significantly higher value of RBC, HGB and HCT than the normal range of the male population (RBC :4.00-5.50 t/l, HGB:120.0-160.0 g/l, HCT: 40-51 %, provided by the statistics in An Zhen Hospital, Beijing). Obviously, this is due to that the dosage used in our experiment is only a clinic recommendation for preventing the volunteers from disaster. If the abuser of rhEPO adopted the recommended dosage and satisfied with their promoted performance the hematological determination would be useless for doping test.

3. As a practical-used procedure it is necessary to distinguish the promoted EPO level by exogenous injection from "the exogenous induction" implying the altitude training. At this moment no evidence has been presented that by biochemical and hematological analysis, altitude training and exogenous rhEPO injection could be told by their difference. Fortunately, the different median charge possessed by endogenous EPO and the recombinant has highlighted the proceeding of EPO test. Either a capillary electrophoresis or its coupling with mass spectrometer would be a outlet for the test, provided that the detection of glycoprotein using electrospray or time of flight mass spectrometer is successful.

REFERENCES

1. Canadian Erythropoietin Study Group, Association between recombinant human erythropoietin and quality of life and exercise capacity of patients receiving haemodialysis *Br. Med. J.* 300:573-578,1990
2. Berglund. B, Effect of recombinant human erythropoietin administration on blood pressure and some haematological parameters in health males *J. Int. Med.* 229:125-130, 1991
3. Ekblom. B and B. Berglund, Effect of erythropoietin administration on maximal aerobic power in man, *Scan. J. Med. Sci. Sports* 11:88-93, 1991
4. Reilly Thomas, "Altitude , Blood Doping and EPO" , *Coaching Focus*, p17-18 No.23 Summer 1993, ISSN 0267 4416
5. Leif Wide et al , " Detection in blood and urine of recombinant erythropoietin administration to healthy men" , Special Communications, *Medicine and Science in Sports and Exercise*, 1995
6. The manual of EPO kit by R&D Systems, U.S.A.
7. The manual of TfR kit by R&D Systems, U.S.A.
8. Martindale p465, Thirtieth Edition , The Pharmaceutical Press, England, 1993
9. T. Salmoson et al, " The pharmacokinetics of recombinant human erythropoietin after intravenous and subcutaneous administration to health subjects" , *Br. J. Clin. Pharmacol.* 29:709-713, 1990
10. Y. Beguin et al, "Quantitative assessment of erythropoiesis and functional classification of anemia based on measurement of serum transferrin receptor and erythropoietin" , *Blood* , 81:1067-1076, 1993