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Diurnal variation of indirect parameters for the detection of recombinant human erythropoietin abuse in athletes

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1 Introduction

Erythropoietin (EPO) is a glycoprotein, that belongs to the haemopoetic growth factors. Recombinant human erythropoietin (rhEPO) is commercially available since 1988. It stimulates the proliferation and maturation of erythroid progenitor cells and thereby increases the number of red blood cells in the identical fashion as human EPO. Repeated injections of rhEPO will increase haemoglobin concentration and haematocrite in a dose and time-dependent manner and therefore enhances the aerobic power of healthy athletes [1].

The abuse of rhEPO is officially prohibited by the International Olympic Committee (IOC) since 1989. In 1999, the International Olympic Committee offered grants to develop reliable methods for detecting the misuse of rhEPO.

The present project is part of a collaboration between the three IOC-accredited laboratories in Oslo, Cologne and Kreischa and an external group (GBF) specialised in EPO-research. The main objectives of the project are first to validate a set of indirect parameters and second to develop a direct method capable of identifying rhEPO with sufficient sensitivity and specificity for application in doping control.

To validate a set of indirect markers it is neessary to investigate the individual variation of indirect parameters under physiological conditions. One part of the project was the investigation of the diurnal variation of EPO.

2 Design and Methods

2.1 Subjects

Sixty-two healthy male (32) and female (30) athletes ranging in age from 18 to 38 years (mean 25,87 ± 4,31 years) were included in the study. The subjects were assigned into two groups: thirty-two well trained volunteers (17 males (6 runners, 5 cyclists and 6 anaerobic sports) and 15 females (5 runners, 5 cyclists and 5 anaerobic sports)) and thirty students in physical education (15 males, 15 females (each group: 5 runners, 5 cyclists and 5 anaerobic

sports)), who were physically active on a recreational basis. All of them had been involved in regular training for several years and during the study period they were allowed to normal physical activity. None of them was taking part in official competition during this period. The characteristics of each group are summarized in Table 1.

Table 1: Characteristics of the subjects

Sex	Group	Age	Body mass (kg)
Female (n=30)	runners (n=10)	28,1 ± 4,93	62,2 ± 6,55
	cyclists (n=10)	25,2 ± 3,88	60,8 ± 5,20
	Anaerobic sports (n=10)	23,8 ± 4,52	66,5 ± 12,37
Male (n=32)	runners (n=11)	26,6 ± 4,84	69,3 ± 5,48
	cyclists (n=10)	25,9 ± 3,73	76,2 ± 7,19
	anaerobic sports (n=11)	25,6 ± 3,61	87,0 ± 16,29

Results are expressed as mean +/- SD

2.2 Inclusion and exclusion criteria

Each subject was submitted to clinical examination before admission to the study. The inclusion criteria of the athletes were age (from 18 to 40 years) and the absence of signs or symptoms of disease. Each volunteer was deemed to be healthy on the basis of normal history, physical examination and electrocardiogram.

Exclusion criteria were age (<18 years); hypertension (blood pressure: systolic >160 mmHg, diastolic >95 mmHg); cardiovascular disease; thrombosis; anaemia; thrombocytosis; blood donors and pregnant women. Subjects were instructed not to consume alcohol or other medications before and throughout the study periode.

Consent: Subjects were fully informed of the procedure and the purpose of the study in advance and their written informed consent was obtained. The study protocol was approved by the Ethics Committee of the German Sport University Cologne.

2.3 Experimental procedure and Sample analysis

Blood samples (9 ml) were drawn from a forearm vein after 5 min of supine rest over a 24 h period to determine whether serum EPO is secreted according to a circadian rhythm. The venous blood samples were taken at 8:00, 12:00, 16:00, 20:00, 23:00 and 8:00 hours. There

was a sleep or rest period from 11 pm to 8 am. Samples were allowed to clot at room temperature and serum was separaed immediately by centrifugation (3400 g at 4°C for 20 min), divided in aliquots and stored at -70°C until analysis. Besides EPO, Ferritin, sTfr and total of protein were determined.

For assessment of haematological parameters (reticulocytes, RBC, Hb, Ht, MCV, MCH and MCHC) blood samples (3ml) were drawn into EDTA coated tubes and stored at 4°C. The blood samples were sent to Kreischa within 24 hours. The analyses were performed immediately using an automated cytoanalyser (Advia 120, haematology analyzer, BAYER DIAGNOSTICS München, Germany).

Serum was analysed for EPO using the NICHOLS Advantage two-site chemiluminescence Erythropoietin immunoassay from NICHOLS INSTITUTE DIAGNOSTIC, Bad Nauheim, Germany. All samples from each volunteer were analyzed in a single-assay run. The results are expressed in mU/mL. The limit of detection was estimated to be 1,2 mU/mL. The assay is referenced to the WHO Recombinant DNA-derived Human EPO 1st IS (WHO 87/684) and can not distinguish between endogenous EPO and rhEPO.

Furthermore ferritin and soluble transferrin receptor levels were determined by using the NICHOLS Advantage specialty system. The serum parameters were measured in Cologne and Kreischa except the total protein level. This parameter was measured only in Cologne using the MIRA PLUS SYSTEM (Abx DIAGNOSTICS, Göppingen, Germany).

2.4 Statistical analysis

Physical characteristics of the subjects are presented as mean ± standard deviation (SD). The statistical analysis was performed using the Statistical Package for Social Sciences (SPSS for Windows, Version 10.0).

The diurnal serum EPO concentration were checked for time-dependency, difference between sex, groups (runners, cyclists and anerobic sports) and trained or well trained athletes by an analysis of variance with repeated measurments using the statistical software package "R" (Version 1.2.1; available *via* internet: http://www.ci.tuwien.ac.at/R) [5]. Statistical significance was considered at $P<0.05$.

3 Results

The individual and mean serum EPO concentrations over a 24 h period are illustrated in Figure 1. The serum EPO concentration showed marked fluctuations during the day. Serum EPO is secreted according to a circadian rhythm with the lowest level at 8 am and the highest

level at 20.00 hours. The mean serum EPO concentration of 32 healthy male athletes was 11.1 ± 3.9 (SD) mU/ml and of 30 women 14.8 ± 8.2 (SD) mU/ml (Tab.2).

There is a significant difference between male and female in this way, that the basic serum EPO values are higher for females as for males (Fig.1 and Tab.2). No significant differences were seen between the amplitude of the curves and there are no signs for a phase-shifting for male or female.

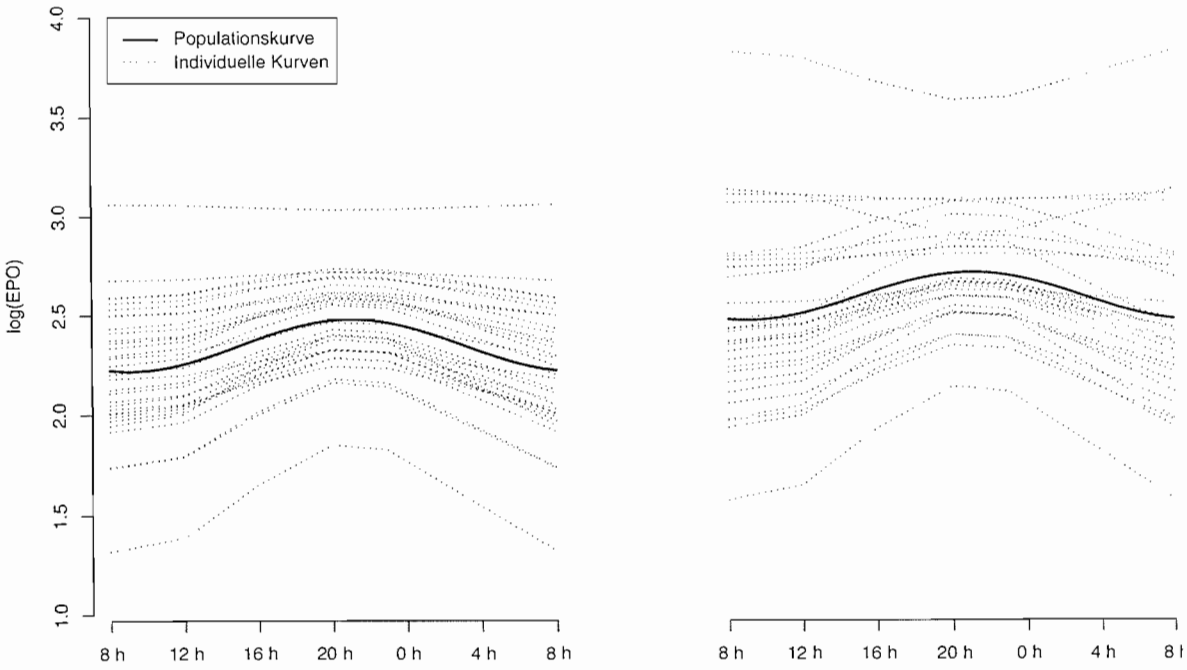

Fig.1: Diurnal fluctuations of individual and population serum erythropoietin (EPO) concentrations over a 24 h period

Further shows Figure 1 that the higher the basic production of the individual serum EPO concentration is the smaller is the amplitude - in extrem cases with reversed premises.
The serum and haematologic parameters were found to be within the clinically normal reference range (Tab.2).

Table 2: Mean serum and haematologic parameters of all subjects

Sex		EPO [mU/ml]	sTfr [nmol/l]	Hematocrit [%]	Reticulocyte Hematocrit [%]	% Macrocytes (> 120 fl)
female n=30	Cologne	14,8 ± 8,2	16,9 ± 4,8	39,5 ± 2,2	0,58 ± 0,2	0,66 ± 1,2
	Kreischa	13,9 ± 6,9	18,4 ± 5,1			
male n=32	Cologne	11,1 ± 3,9	17,1 ± 4,1	43,2 ± 3,6	0,64 ± 0,2	0,39 ± 0,5
	Kreischa	10,9 ± 3,8	18,0 ± 3,9			

Results are expressed as mean +/- SD

All serum samples were analysed for EPO, Ferritin and soluble transferring receptor (sTfr) with the same ELISA in Cologne and Kreischa. One aim of this study was to compare these results. Figure 2 shows for EPO that the results in both laboratories are comparable.

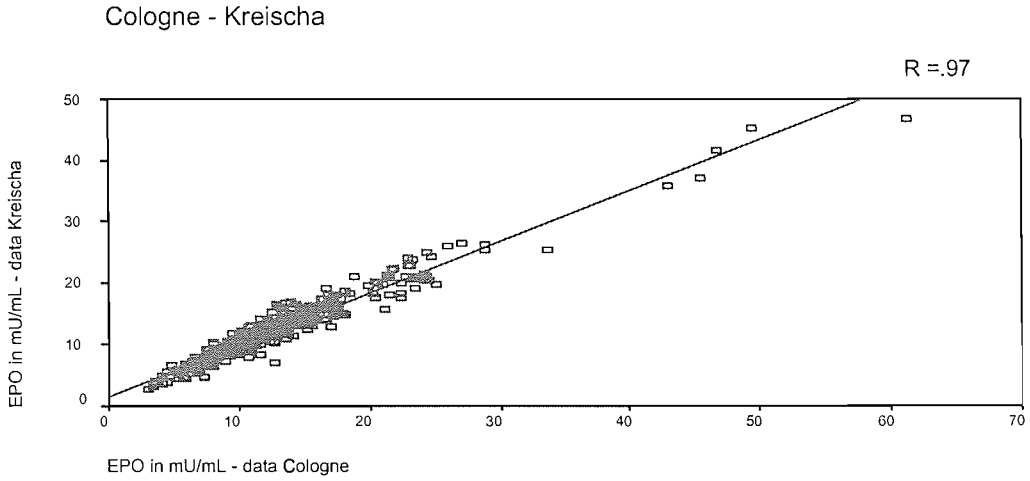

Fig. 2: Comparison Cologne – Kreischa

4 Discussion

As previously documented by Wide (1989), the present study confirms the existence of a well defined circadian rhythm of serum EPO levels in healthy subjects, with its maximum at 20:00 hours.

The renal EPO production depends on the oxygen partial pressure in the involved tissues. Change in venous pO_2 regulates the EPO production. The reduced metabolism during the night results in a lower oxygen requirement of the involved tissues and thereby leads to a higher venous pO_2. That could be the reason for the decrease in serum EPO concentration during the night.

It is further hypothesized that several of the hormones (for example: Cortisol, ACTH, testosterone and T3, T4) secreted or regulated by the pituitary gland show circadian rhythms and they or the variations of blood flow through the kidney during the day may be involved in the control of the observed circadian rhythm of EPO [3,12]. Grünefelder (1996) mentioned that the erythroid progenitor cells in the bone marrow utilize serum EPO periodically and causes so the fluctuation of serum EPO concentration.

The total collection of 72 ml blood for each volunteer may have in itself stimulated EPO production. However, the mechanisms behind the observed circadian rhythm of serum EPO and the interpretation of the experimental data is equivocal. Unambiguous is the fact that there is an circadian rhythm of serum EPO.

The comparison between the measured serum EPO concentrations in Cologne and Kreischa showed good accordance (Fig. 2). This result concludes a defining analytical robustness for EPO measurment in both laboratories.

References

[1] Berglund, B.; Ekblom, B.: Effect of recombinant human erythropoietin treatment on blood pressure and some haematological parameters in healthy men. J. of Internal Medicine (229): 125-130,1991.

[2] Casale, R.; Pasqualetti, P.: Diurnal rhythm of serum erythropoietin circulating levels in chronic obstructive pulmonary disease. Panminerva Medica 39 (3): 183-185, 1997.

[3] Cotes, P.M.: Immunoreactive erythropoietin in serum. Br J of Haematology 50: 427-438, 1982.

[4] Grünefelder, CH.: Circadian periodicity of serum erythropeietin. Schweizerische Rundschau für Medizin (Praxis) 85 (41): 1283-1293, 1996.

[5] Ihaka, Ross and Roberts GENTLEMAN: R: A language for data analysis and graphics. J. of Computational and Graphical Statstics, 5 (3): 299-314, 1996.

[6] Klausen, T., et al.: Diurnal variations of serum erythropoietin at sea level and altitude. Eur. J. Appl. Physiol. 72 : 297-302, 1992.

[7] Klausen, T.; Dela, F. ; Hippe, E.; Galbo, H.: Diurnal variation of serum erythropoietin in trained and untrained subjects. Eur. J Appl Physiol 67 (6) : 545-548, 1993.

[8] Parisotto, R.; Wu, Moutian; Ashenden, M.J.; Emslie, K.R.; Gore, C.J.; Howe, C.; Kazlauskas, R.; Sharpe, K.; Trout, G.J.; Xie, Minhao; Hahn, A.G.: Detection of recombinant human erythropoietin abuse in athletes utilizing markers of altered erythropoiesis. Haematologica 86 (2): 128-137, 2001.

[9] Pasqualetti, P.; Casale, R.: Circadian rhythm of serum erythropoietin in healthy subjects. European Review for Medical and Pharmacological Sciences 18: 91-93, 1996.

[10] Pasqualetti, P.; Casale, R.: No influence of aging on the circadian rhythm of erythropoietin in healthy subjects. Gerontology 43 (4): 206-209, 1997.

[11] Roberts, D.; Smith, D.J. : Erythropoietin does not demonstrate circadian rhythm in healthy men. J. Appl. Physiol. 80 (3): 847-851, 1996.

[12] Wide, L.; Bengtsson, C.; Birgegard, G.: Circadian rhythm of erythropoietin in human serum. Br J. of Haematology 72: 85-90, 1989.