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Individual Reference Ranges for serum Erythropoietin (sEPO)- Part II: Influence of Long Distance Flying

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1 Introduction

Erythropoietin (EPO) is a glycoprotein, that belongs to the haemopoetic growth factors. The fetal EPO is synthesized in the liver and after birth in the kidney. The renal EPO production depends on the oxygen partial pressure in the involved tissues and is influenced mostly by the amount of red blood cells (RBC). Hyperoxia suppresses EPO formation, whereas hypoxia leads to an enhancement of EPO production [10, 6]. The endogenous serum erythropoietin (sEPO) levels of healthy individuals range between 10 and 25 mU/mL, the urinary EPO between 2-5 mU/mL [1,2,3].

Gunga [8] mentioned that the cabin pressure during a flight in a pressurized aircraft is equal or below an elevation of 8000 ft (2438 m) and causes an arterial oxygen saturation of at least 90%. He found that these conditions elevate sEPO significantly.

One aim of this present study is to investigate whether a long distance flight might elevate endogenous sEPO levels above individual or population based reference ranges. Therefore a total of 11 healthy, untrained to medium trained subjects (6 males/ 5 females) were studied during two regular long-distance flights which were 76 hours apart.

Another aim is to compare two chemiluminscences immunometric assays from different manufacturers (a manual vs an automated assay).

2 Materials and Methods

2.1 Reagents

Serum erythropoietin levels were determined by using chemiluminescence erythropoietin immunoassays from NICHOLS INSTITUTE DIAGNOSTIC, Bad Nauheim, Germany and DPC Biermann GmbH, Bad Nauheim, Germany. The immunoassay from NICHOLS is a manual assay in contrast to the fully automated „IMMULITE“ system from DPC.

Both immunoassays are sandwich solid-phase assays. NICHOLS uses a one-step incubation with a primary polyclonal sheep antibody to recombinant human EPO, which is coupled to biotin. The second antibody is a monoclonal mouse antibody, which is labeled with an acridinium ester for detection. With this marker light emission is triggered by an alkaline oxydation and the chemiluminescence reaction is counted by a luminometer. The concentration of EPO in the sample is directly proportional to the amount of bound labeled antibody and therefore to the emited light. The sensitivity of this assay is 1.4 mU/ml as calculated by NICHOLS INSTITUTE DIAGNOSTICS. The assay was carried out according to the user manual with the exception that each standard solution was measured in triplicate and all unknown samples were measured once.

The accuracy, precision and specificity of the NICHOLS INSTITUTE Diagnostics chemiluminescence immunoassay were determined in an earlier study [2]. The inter-assay variance was determined over four runs with three QC samples with mean concentrations of 11.6, 26.4 and 47.7 mU/mL and coefficients of variation (CV) of 9.3%, 7.0% and 8.5% respectively.

For the DPC-Bierman assay the primary antibody is a murine monoclonal antibody to recombinant human EPO. In a first incubation step EPO binds to the primary antibody. An alkaline phosphatase-labeled polyclonal goat antibody is added in a second step. The free labeled antibodies are removed by a centrifugal washing step. The alkaline phosphatase hydrolyzes the substrate adamantyl dioxetane phosphate ester. This reaction leads to emission of light, which is measured in the integrated luminometer. The analytical sensitivity of this assay is 0,24 mU/mL as calculated by DPC. The sample volume is 100 µL serum as with the NICHOLS assay but there is a void volume of an additional 130 µL.

The IMMULITE assay uses a master calibration predifined by the manufacturer. Each new kit has to be adjusted to the specific enviroment by measuring a low and high adjustor included in the kit. The inter-assay variance was determined over four runs with three QC samples with mean concentrations of 12.3, 33.2 and 55.2 mU/mL and coefficients of variation (CV) of 4.8%, 6.8% and 7.8% respectively.

2.2 Study design

A total of 11 healthy, untrained to medium trained subjects (6 males/ 5 females) were studied during two regular scheduled long-distance flights which were 76 hours apart. During the flights, the cabin pressure was equivalent to an altitude of 1800-2000 m above sea level.

Venous blood samples were withdrawn 25 h and 7 h before, 12, 24, 48 and 72h after the first take off (samples 1 to 6); and 5h before 11,18, 31,55 and 79 hours after the second take off (samples 7 to 12). The first flight lasted 7 hours on final cabin pressure, the second one 5.5 hours.

2.3 Blood samples

Blood samples were withdrawn from a forearm vein and collected into serum and EDTA sample tubes (Sarstedt, Nümbrecht, Germany and Becton Dickinson Vacutainer Systems, Heidelberg, Germany). For each sampling 10 mL of blood were collected in serum tubes and for samples 1 and 8-12 also 3 mL of blood were collected in EDTA sample tubes. From each subject a total amount of ca. 140 mL blood were withdrawn. During the withdrawal the subjects were lying flat.

The samples were allowed to clot at room temperature for 15 to 30 min. and serum was immediately separated by centrifugation at 1500 g for 10 min at room temperature. The serum samples were put on dry ice right away and later stored at -70° C until analysis.

2.4 Calculations

The results were calculated using the statistic software package „S-Plus 3.4" from MathSoft Inc., Cambridge, MA, USA running on an IBM RS6000-990 under AIX 4. A four-parametric logistic model was fitted to the calibration data using the S-Plus function „Calibration" from O'Connell, M.A. et al. [11]. The concentrations of the unknown samples were calculated using the S-Plus function „Multi.Calib" from Robinson-Cox, J.F.[12]. These functions are available from the StatLib-Server (http://lib.stat.cmu.edu).

Since sEPO values do not follow a Gaussian distribution, results are expressed as medians ± median absolut deviation where applicable. Median absolult deviation is a robust equivalent to standard deviation [9, pp. 308]. The Kruskal-Wallis test was used for analysis of variance of sEPO levels.

3 Results

3.1 Influence of the long distance flights

The time courses of sEPO, as determined with the NICHOLS kit, are shown in Figure 1. During the flight, the cabin pressure was equivalent to an altitude of 1800-2000 m above sea level. Of the 11 subjects, three showed values above the 99th percentile of a distribution of

sEPO levels of 229 elite athletes (unpublished data). One is a female on the verge of anemia, the second is a male who returned from a one week stay between an altituted of 1800 - 3300 m just two weeks before the beginning of the study. The high values of the third subject is caused by a naturally elevated sEPO [3]. The sEPO level of the other 8 subjects are below the 99th percentile of a distribution of sEPO levels of 229 elite athletes.

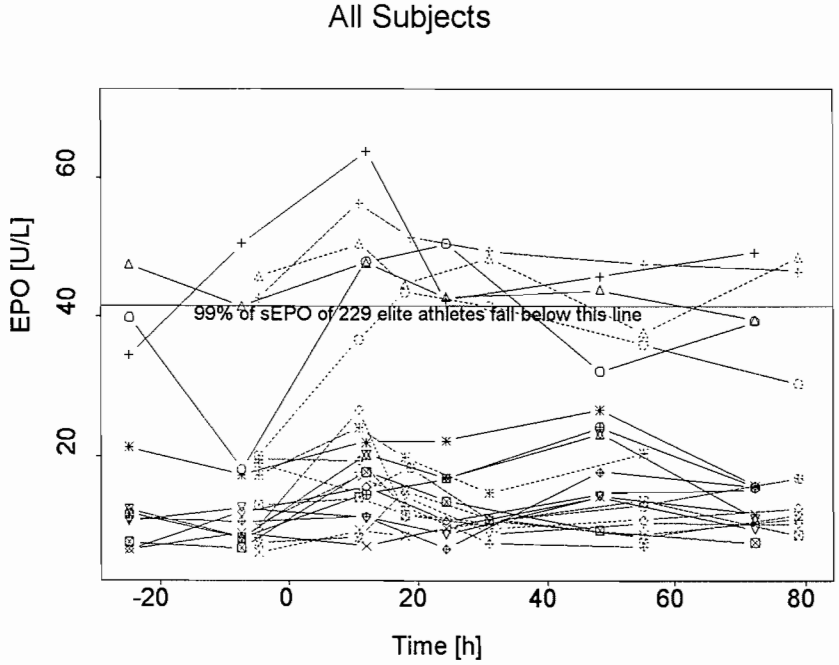

Figure 1: Time courses of endogenous sEPO levels

Ten of the eleven subjects were part of an earlier study in January 1998 [3]. Those results were used to set up individual reference ranges. Seven of the 10 individuals stayed within the mentioned reference ranges concerning the two basline values taken before the first take off. Two of the remaining subjects showed values above their individual reference ranges and one subject fell short of it (data not shown).

The time courses of sEPO didn't show a uniform trend at all. The individual levels of a few subjects rose reaching peak concentrations 12, 24 or 48 h (respectively 11, 18, 31 or 55 h) after take off and some sEPO time courses were so irregular that no trend can be described (data not shown). But none of the subjects came close to the sEPO levels measured 48 h after a single subcutaneous application of 100 I.U. rhEPO per kg bodyweight [3].

Table 1 shows the median sEPO concentrations for the different sampling times. Analysis of variance (Kruskal-Wallis test) for these different sampling times was not significant ($p > 0.05$).

Tab. 1: sEPO levels at different sampling times

sample	sampling time	sEPO (median ± mad)
1	- 25 [(1)]	11.7±7.7
2	- 7 [(1)]	11.8±5.3
3	+ 12 [(1)]	17.7±9.6
4	+ 24 [(1)]	16.7±10.5
5	+ 48 [(1)]	23.2±13.3
6	+ 72 [(1)]	15.5±6.5
7	- 5 [(2)]	17.2±10.7
8	+ 11 [(2)]	19.4±10.7
9	+ 18 [(2)]	15±5.5
10	+ 31 [(2)]	10.7±2.8
11	+ 55 [(2)]	12.8±6.7
12	+ 79 [(2)]	12.5±5.8

[(1)] Samples of the first flight
[(2)] Samples of the second flight

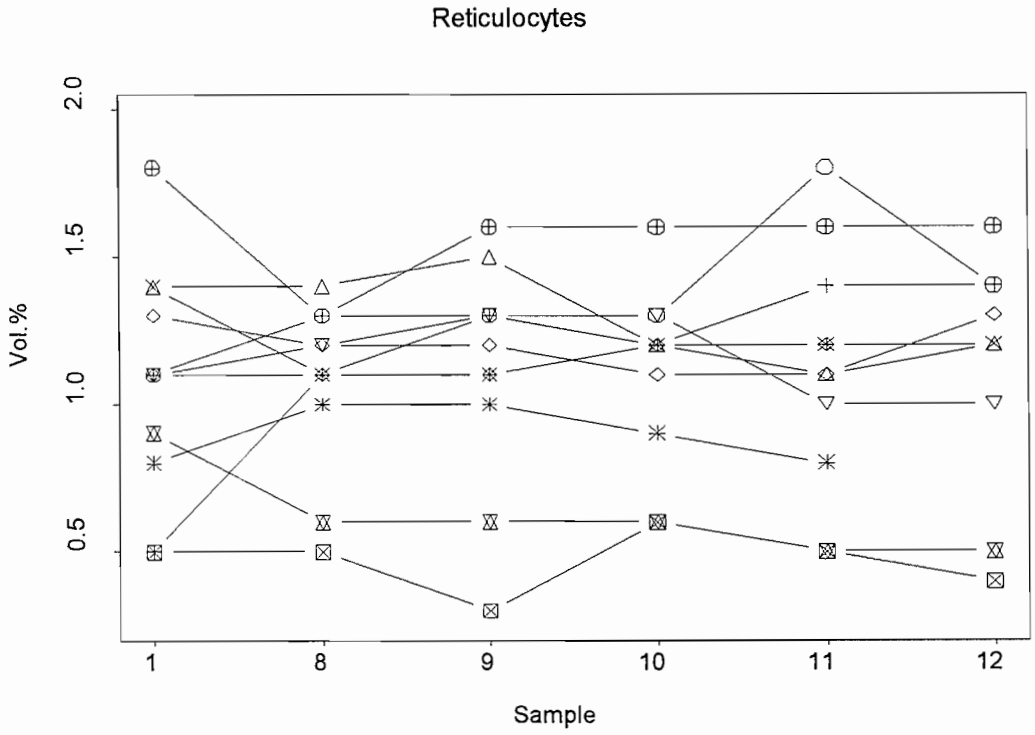

Figure 2: Reticulocytes concentrations of 11 healthy subjects before and after two long distance flights

Tab. 2: Reticulocytes level at different sampling times

sample	Reticulocytes (mean± stdev)
1	1.08 ± 0.39
8	1.07 ± 0.28
9	1.12 ± 0.38
10	1.11 ± 0.3
11	1.11 ± 0.41
12	1.11 ± 0.41

Figure 2 shows the distribution of reticulocyte concentrations in samples withdrawn before the first take-off and after second take-off (samples 8-12). Table 2 shows the reticulocyte concentrations at the different sampling times, which were all within the reference range (0.8 - 2.5 Vol% M; 0.8 - 4.1 Vol% F) [4].

3.2 Comparison of the two immunoassays

Figure 3 shows the comparison of 6 samples between the NICHOLS and the DPC kit. Samples were analyzed undiluted (DPC), two fold diluted (DPC/NICHOLS) and four fold diluted (NICHOLS). The dilutions were carried out according to the manufacturers suggestions. The bars show the corrected concentrations.

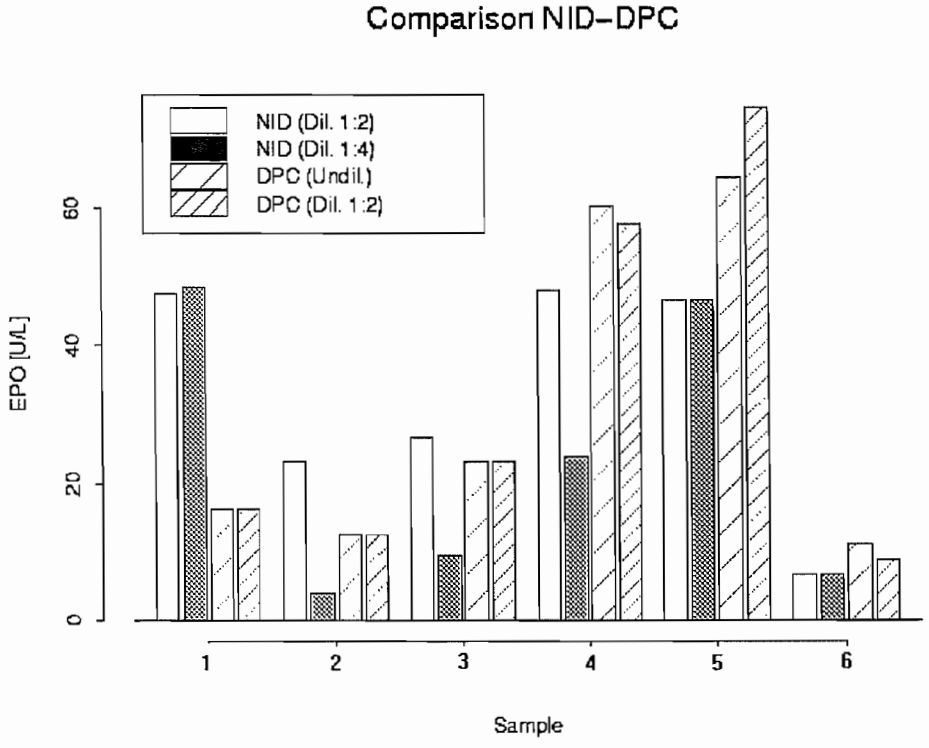

Figure 3: Comparison NID-DPC assay

4 Discussion

The aim of the present study was to investigate whether a long distance flight might elevate endogenous sEPO levels above individual or population based reference ranges. The results didn't indicate a clear trend at all. Most of the subjects displayed a time course that didn't show the expected increase after the flight with a subsequent decrease of sEPO levels. For a few subjects one or two samples were outside their individual reference range and those samples were not necessarily the ones withdrawn after the flights. Overall the patterns of the sEPO time courses were very irregular.

Gunga et. al. [8] reported sEPO levels of 7 crew members of a routine military flight before, during and after an eight hour flight with a cabin pressure equivalent to ca. 2400m above sea level. He reported baseline values of 10.4±4.4 U/L (mean ± SD), that rose significantly to 23.1 ± 7.3 U/L 7 hours into the flight and fell to 15.0 ± 10.3 U/L 8 hours after landing (16 hours after take-off). This time course of sEPO values doesn't accord with the results of sEPO levels determined in the present study. We measured similar sEPO levels before the flights and sometime after the flights (f.i. samples 4 and 9, Tab.1), but we weren't able to show a significant rise just after the flights. However the cabin pressure was equivalent to an altitude of 1800-2000 m, which is less than the altitude reported by Gunga.

Even though there is obviously no significant effect on erythropoesis, a few subjects showed sEPO values outside their individual reference ranges. In our opinion two factors contribute to this. Firstly, the individual reference ranges were based on twelve samples per individual taken on two consecutive days. This is certainly too short a time periode to record most of the individual variations. Secondly, the determination of sEPO itself is rather problematic.

The comparison between the DPC and the NICHOLS kit showed good accordance for most of the samples. But for a few samples the results differed widely. Even dilution of the samples didn't resolve these discrepancies. For instance, for one of the subjects, all the results of the NICHOLS kit were well above his individual reference range, previously determined in another study with the NICHOLS kit (data not shown). But the results of the DPC kit were all but four within that reference range. This suggests that unusual results have to be verified by a second and different reagent kit (antibody) before being accepted. Also, this stresses the importance of using adequate quality control samples to check the performance of the reagents to exclude that outlying results are due to poor performance. In our case, both kits performed well according to QC data. Following the principle "In dubio pro reo", the differing results would then have to be interpreted in favor of the subject.

Population based reference ranges are rather wide by nature, which means that missuse with small dosages might go undetected. A narrower filter would be an individual reference range. The strength of such a range depends of course on the way the data is collected. The twelve samples collected over two consecutive days from the earlier study that we used as references for the results of this study obviously did not reflect the individual variation sufficiently.

Concluding, we couldn't demonstrate that the hypoxic stress due to the low cabin pressure (equivalent to an altitude of 1800 - 2000 m) during a regular scheduled long distance flight significantly increases erythropoietic activity of the bone marrow. Neither the sEPO level nor the amount of reticulocytes rose significantly (Fig. 1 and 2). Three subjects mentioned in section 3.1 showed values above a population based reference range (99th percentile) of sEPO levels of 229 elite athletes. For all of those subjects, a reasonable explanation could be given. To obtain valid individual reference ranges, the samples have to be collected over a longer period of time considering different factors possibly effecting erythropoesis. Unusual results of one immunoassay have to be validated by a second independent immunoassay.

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