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Physiological effects of intermittent hypoxia in triathletes and detection of EPO abuse

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Introduction

Adaptation to hypoxia stimulates endogenous erythropoietin (EPO) secretion, an anti-apoptotic hormone necessary for the differentiation of erythroid precursors into mature red cells, as a reaction to improve oxygen transport capacity by increasing the red blood cell mass [1]. Different altitude training modalities have proliferated in order to get advantage of the natural EPO stimulation, trying to enable athletes to optimise both the stimuli necessary to improve oxygen delivery and utilization while avoiding the detraining effects associated with chronic hypobaric hypoxia [2-4]. Among them, a promising technique for administering short-term hypoxia combined with sea-level training, termed "intermittent hypoxia exposure" (IHE), has been proposed based on the observation that brief exposures to relatively high levels of hypoxia stimulate the release of EPO in humans [5-8].

Similar results can be artificially obtained by the administration of recombinant human erythropoietin (rhEPO) or its analogues (i.e. darbepoetin, NESP) [9, 10]. Their use is banned by the World Anti-doping Agency (WADA) as well as other major sports authorities and two different approaches have been developed to detect their abuse. The direct identification method, currently supported by the WADA, is performed in urine samples and is based on the differences of isoelectric profile between the exogenous rhEPO and endogenous EPO [11], that are supposed to be mainly reflecting differences in glycosylation. However, no previous

study has investigated how final glycosylation of urinary EPO could be modified when the production of EPO is increased dramatically under hypoxic exposure.

The indirect methods, developed to indicate current (ON-models) or recently discontinued (OFF-models) rhEPO administration, consist of mathematical models which use combinations of haematological parameters sensitive to variations in erythropoiesis [12]. These models have been previously applied to real and simulated altitude studies [13, 14]; however, results reinforce the notion that caution should be exercised when interpreting blood results from athletes who have recently been exposed to either terrestrial or simulated altitude.

The objectives of the present work were to study how the adaptation to intermittent hypoxic exposure (IHE) in well trained athletes would affect athletic performance and haematological parameters, and how this physiological adaptation to IHE could affect the parameters involved in the interpretation of methods (direct and indirect) currently used to evaluate potential recombinant human EPO (rhEPO) misuse.

Methodology

Sixteen healthy, highly trained male triathletes (mean age 27±6 years old) were randomly assigned to either hypoxic (HYPO, n=8) or normoxic (NORM, n=8) groups. The HYPO group combined training at sea level with resting exposure to hypoxia in a hypobaric chamber for 3 h/day, 5 days a week, for 4 weeks (Figure 1). Simulated altitude progressively increased from 4,000 up to 5,500 m (from 614 to 504 hPa) during the first four sessions and was maintained at 5,500 m in the subsequent sixteen sessions. The NORM group remained all time in ambient normobaric conditions. Athletes were supplemented with oral iron and antioxidant vitamins to ensure an adequate status during the protocol. The protocol was approved by the Instituto Municipal de Asistencia Sanitaria Ethics Committee of Clinical Research (CEIC/IMAS nº. 2000/1145/I) and was in accordance with the Helsinki Declaration. All the subjects were informed and gave written consent to participate.

Physical performance was measured on different occasions (see Figure 1) using two tests: a graded running test on a synthetic 400-m track, in which velocity at VO_2max or maximal aerobic speed was determined; and a maximal, incremental test on a running treadmill with continuous monitoring of gas exchange parameters. Serum, EDTA blood and urine samples were collected on two occasions from both groups: immediately before the first chamber exposure (t_1) and immediately before the last exposure (t_{26}). A third sample' collection was

carried out to follow the HYPO group return to continuous normoxic conditions (t_{39}). Finally, to study the acute effect of hypobaric hypoxia in the HYPO group, samples were collected on two more occasions: 3 hours after the first ($t_{1'}$) and the last ($t_{26'}$) exposures.
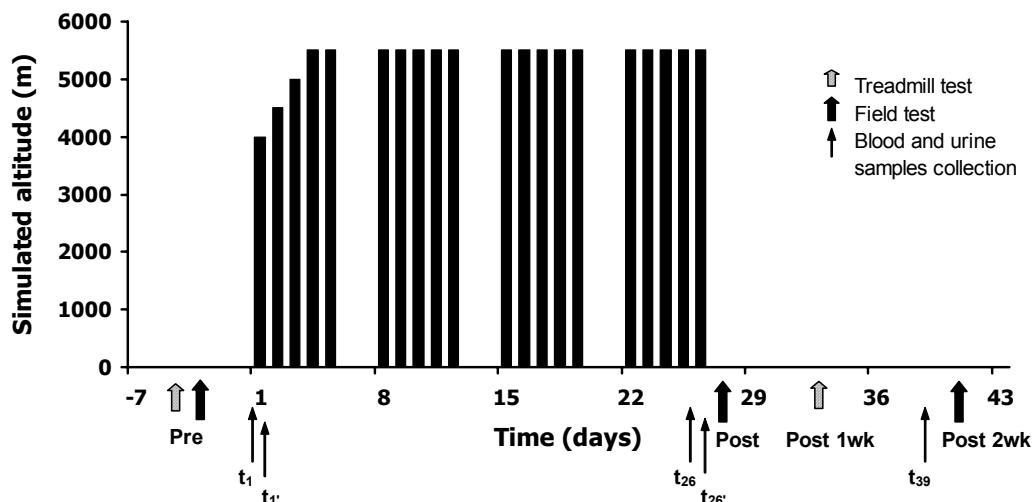

Figure 1. Experimental protocol of exposure to intermittent hypoxia in the hypobaric chamber.

Blood cell counts and reticulocyte parameters were analysed using a Sysmex XE-2100 autoanalyser (Roche Diagnostics, Mannhein, Germany). Serum EPO and soluble transferrin receptor (sTfR) concentrations were measured using immunoassays (Quantikine IVD Human EPO, R&D Systems, and Tina-quant sTfR, Roche Diagnostics, respectively, using a Hitachi 911 autoanalyzer).

Second-generation mathematical models (ON he, ON hes, OFF hr and OFF hre), developed by Gore et al. [12], use concentrations of hemoglobin (h), erythropoietin (e), soluble transferrin receptor (s), as well as percent reticulocytes (r). As the immunoassays used to measure serum EPO and sTfR were different from those used in the development of those models, those values were transformed using the inter-technique correlation equations calculated in previous studies [15, 16].

The method used as urinary EPO test (comparison of EPO isoforms by isoelectric focusing) was based on that described by F. Lasne et al. [11]. The criteria used for the evaluation of the presence of the recombinant substances (rhEPO and NESP) was based on the co-localization of the most intense bands (isoforms of urinary EPO) in the area corresponding to those equivalent for the exogenous standards, as described in the WADA Technical Document [17].

Accordingly, we studied all through the protocol, any trend of the urinary EPO isoforms towards those co-localized with isoforms of the corresponding rhEPO standard (basic area) in each volunteer.

Multiple analysis of variance (MANOVA) and post-hoc Student test were carried out using the statistical package SPSS 2001 for Windows, v.11.0.1 (SPSS Inc., Chicago, IL, USA).

Results

Physical performance results are listed in Table 1. Twelve subjects (HYPO n=5 and NORM n=7) successfully completed the performance protocol. Before the chamber sessions, no differences were found between the two groups. HYPO group significantly increased their ventilatory threshold (+3.8%, $p<0.001$) and reduced significantly their CO_2 production at maximal effort (-8.6%, $p=0.02$), as compared with the NORM group. However, we could not detect a significant difference in the change of VO_2max within or between groups although a trend was observed in the HYPO group (+3.7%, $p=0.2$), nor an improvement in running performance in the track.

Table 1: Performance variables in triathlon athletes during the experimental protocol.

	PRE		POST		POST 1wk		POST 2wk	
	NORM	HYPO	NORM	HYPO	NORM	HYPO	NORM	HYPO
MAS (km/h)	16.0±0.8	16.4±1.3	15.8±0.5	16.4±1.3	-	-	16.0±0.8	16.4±0.9
VO_2max (mL/kg·min)	60.3±9.5	58.8±4.6	-	-	60.9±5.9	61.0±4.4	-	-
VO_2max (mL/min)	4563±793	4330±496	-	-	4569±571	4399±483	-	-
VCO_2max (mL/min)	5465±804	5345±390*	-	-	5465±804	5063±546	-	-
$AT1_{vent}$ (%VO_2max)	80.5±5.6	79.8±3.9	-	-	78.5±5.7	83.3±3.1	-	-
$AT2_{vent}$ (%VO_2max)	94.5±2.7	89.1±2.3	-	-	92.4±2.0*	91.6±2.9	-	-

Values are means ± SD. *$p < 0.05$ different from the respective pre conditions. MAS, maximal aerobic speed; VO_2max, maximal O_2 uptake; VCO_2max, maximal CO_2 production; AT_{vent}, ventilatory aerobic threshold.

Haematological variables and erythropoietic serum markers of triathlon athletes are listed in Table 2. Fourteen men (HYPO n=6 and, NORM n=8) successfully completed the sample collection protocol. Before the chamber sessions (t_1), no difference was found between the two groups. In the HYPO group, a significant increase in the level of serum EPO was observed after chamber sessions ($t_{1'}$ vs. t_1 and $t_{26'}$ vs. t_{26}). Small but significant differences were also found in sTfR ($t_{1'}$ vs. t_1 and $t_{26'}$ vs. t_{26}) and %ret ($t_{1'}$ vs. t_1). Between first and last chamber sessions (t_{26} vs. t_1), serum EPO levels decreased in the HYPO group, but not in the NORM group. Moreover, the levels of Hb and immature reticulocyte fractions showed significant differences, but these differences occurred in both groups.

Table 2: Haematological variables and erythropoietic serum markers in triathlon athletes during the experimental protocol.

|	NORM t_1	HYPO t_1	HYPO $t_{1'}$	NORM t_{26}	HYPO t_{26}	HYPO $t_{26'}$	HYPO t_{39}
Hb (g/dL)	15.0±1.3	14.6±0.3	14.3±0.8	14.3±1.1$^+$	13.9±0.4$^+$	14.3±0.7*	13.5±0.5$^+$
RBC ($\times 10^{12}$/L)	5.0±0.5	4.9±0.2	4.8±0.3	4.7±0.3	4.6±0.2$^+$	4.7±0.3*	4.4±0.2$^+$
Hct (%)	44.1±2.9	44.5±2.9	42.8±2.0	42.7±3.0	41.0±1.5	42.0±2.2	39.6±1.7$^+$
%ret (%)	0.8±0.2	0.8±0.2	0.9±0.2*	1.0±0.3$^+$	0.7±0.2	0.7±0.2	0.8±0.2
IRF (%)	9.1±3.0	9.7±3.3	10.7±4.3	2.9±1.4$^+$	1.5±0.9$^+$	2.1±1.1$^+$	3.0±1.5$^+$
sTfR (mg/L)	3.5±0.3	3.5±0.4	3.3±0.3*	3.3±0.4	3.1±0.3	3.2±0.4*$^+$	2.9±0.3$^+$
EPO (IU/L)	10.7±3.1	8.3±3.2	16.6±4.7*	10.8±2.8	4.6±1.4$^+$	24.8±9.3*$^+$	9.2±3.2$^+$

Values are means ± SD. $^+$p < 0.05 different from the initial value for each group (t_1); *p < 0.05 different from the respective pre-chamber exposure value (t_1, t_{26}). Hb, haemoglobin; RBC, red blood cells, Hct, haematocrit; ret, reticulocytes; IRF, immature reticulocyte fractions; EPO, erythropoietin; sTfR, soluble transferrin receptor.

Individual scores of triathlon athletes for each model are represented in Figure 2. All athletes' scores were below the threshold corresponding to 1:100 (random chance for one false positive in one hundred cases) for ON models and 1:1000 for OFF models [12]. However, two volunteers, one from the NORM and one from the HYPO group, obtained relatively high individual scores (located above the 1:10 threshold) in some of the ON/OFF models. The NORM volunteer showed the highest haemoglobin concentration values (17.5 and 16.7 g/dL

in t_1 and t_{26}, respectively); and the HYPO volunteer showed, at $t_{26'}$, a high value of haemoglobin concentration (15.4 g/dL) and the highest EPO concentration observed after chamber exposure (52 IU/L).

Regarding the urinary EPO isoforms analysis, none of the samples studied were suspected to be positive taking into account the criteria previously mentioned [17]. However, the percentage of basic isoforms, taken as a measure of the shape of the profiles, showed a significant increase under the effect of the IHE protocol but only at $t_{26'}$ with a subsequent return to baseline levels at t_{39} (see Figure 3 for a representative result and Table 3).

**Figure 2**: Plot of individual scores for the two ON and two OFF models versus time.
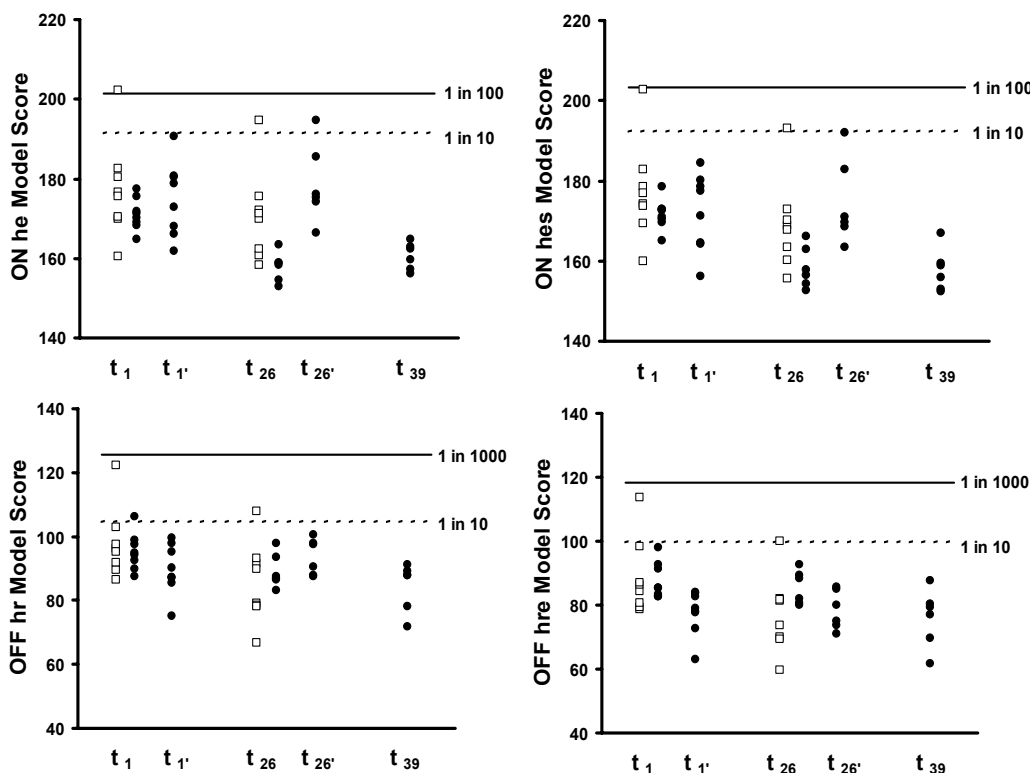
The lines represent the threshold values corresponding to selected false-positive rates for worst-case male endurance athletes at sea level (1 in 10 for the ON/OFF models (dashed lines); 1 in100 for the ON models and 1 in 1000 for the OFF models (solid lines)) defined by Gore C.J. et al. [12]. Empty squares represent NORM group individual scores; full circles represent HYPO group individual scores.

Discussion

The exposure to intermittent hypobaric hypoxia (IHE) has been related to an increase in circulating serum EPO, usually followed by an increase in red blood cell mass [13, 18, 19].

However, our study in highly trained athletes suggests that this is not always true [20]. The main change observed after hypobaric IHE was an increase in serum EPO that did not seem to cause an effective erythropoietic response. Erythroid parameters, such as Hb, reticulocytes and serum transferrin receptor, were not modified despite the serum EPO concentration increase. Moreover, the lack of improvement in running performance is consequent with the non-observed effects over the erythropoietic system. Similar findings have been showed by previous studies in which different protocols of simulated intermittent hypoxia were assessed and no erythroid response was observed [21, 22].

Figure 3: IEF gel with rhEPO and NESP standards and samples from one of the athletes exposed to IHE.
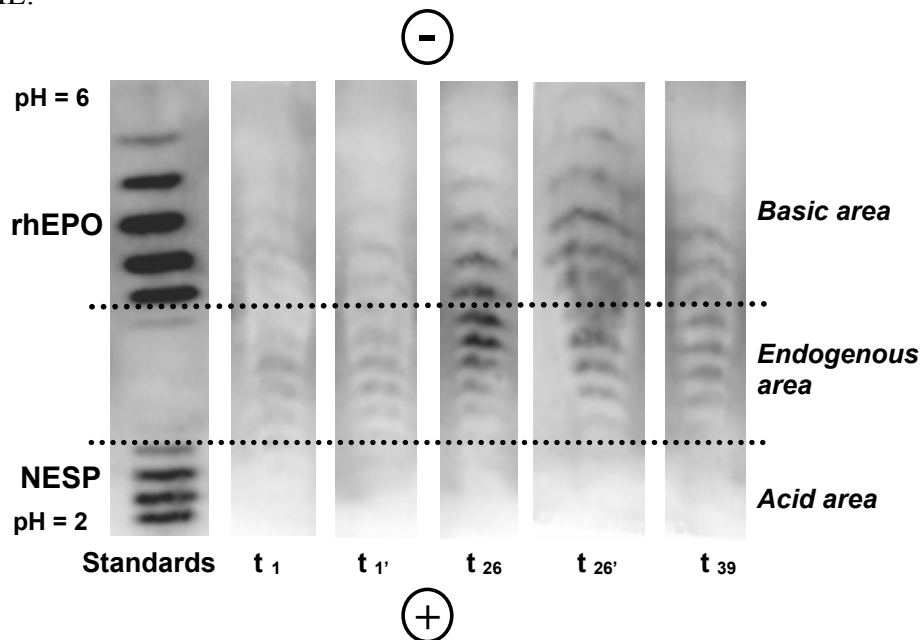
Regarding the direct method for detection of rhEPO misuse, in our conditions, stimulation of the EPO secretion produced by the IHE chamber treatment did not significantly alter the isoelectric profile of the natural protein in urine, as measured by the percent basic isoforms. Modifications in the urinary EPO glycan chains could result from the increased production rate of EPO caused by hypoxia. Therefore, the resulting modified glycoform population of urinary EPO could potentially lead to a decreased electric negative charge, more similar to the rhEPO isoelectric profile. Less negatively charged serum EPO isoelectric profile has been observed in healthy newborn infants and patients with polycythaemia vera compared to healthy adults [23]. The only study published on EPO electrophoretic mobility after hypoxic stimulation [24] reported increased serum EPO levels but no differences in the electrophoretic

mobility of EPO isoforms under short-term and intermittent normobaric hypoxia exposure, from those in normoxic conditions. Our results indicated that the repeated hypoxic exposure followed in this protocol induced not only an increase in EPO concentration but also after several weeks of repeated exposure, a consistent shift towards more basic isoforms. Nevertheless, all those samples were always well evaluated as negative according to the criteria applied.

Table 3: Percent of basic bands obtained for 8 triathlon athletes exposed to intermittent hypobaric hypoxia (HYPO group) during the experimental protocol.

Volunteer	t_1	$t_{1'}$	t_{26}	$t_{26'}$ [+*]	t_{39}
1	36.8%	35.8%	36.0%	58.7%	34.4%
2	19.9%	38.8%	ND	24.4%	19.9%
3	42.4%	47.1%	40.8%	59.3%	NS
4	12.4%	23.1%	21.8%	37.1%	18.6%
5	29.8%	21.3%	ND	61.6%	15.5%
6	26.0%	21.6%	28.3%	50.1%	47.9%
7	29.7%	39.4%	NS	NS	NS
8	29.8%	25.8%	NS	NS	NS

[+]$p < 0.05$ different from the initial value (t_1); *$p < 0.05$ different from the pre-chamber exposure value (t_{26}).

Abbreviations: ND, below the limit of detection; NS, no sample available.

The IHE modified the final score when mathematical ON and OFF models for indirect detection of rhEPO misuse were applied, but never enough to produce a false positive interpretation. Regarding to the score thresholds, only one triathlete from the NORM group in our study exceeded the 1 in 100 threshold; whilst it has been shown that 50-80% of athletes exceed this level during treatment with various doses of rhEPO [12].

In summary, the IHE chamber protocol studied (4 wks, 3 h/d, 5 d/wk at 4,000-5,500 m) produced a moderate increase in EPO serum concentrations, but did not produce any erythroid response nor a performance enhancement. Moreover, no false positive interpretation for EPO abuse using the isoeletric focusing direct method in urine or the indirect method based on second-generation ON/OFF models in blood was observed.

Acknowledgements

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