

**Increased serum erythropoietin but not red cell production after 4 wk of intermittent hypobaric hypoxia (4000 – 5500m)**

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**Abstract**

This study tested the hypothesis that athletes exposed to 4 wk of intermittent hypobaric hypoxia exposure (IHE 3h/d, 5d/wk at 4000-5500m) or double-blind placebo increase their red blood cell volume (RCV) and hemoglobin mass ( $Hb_{mass}$ ) secondary to an increase in erythropoietin (EPO). Twenty-three collegiate level athletes were measured before (PRE) and after (POST) the intervention for RCV via Evans blue (EB) dye, and in duplicate for  $Hb_{mass}$  using CO-rebreathing. Hematological indices including EPO, soluble transferrin receptor (sTfr) and reticulocyte parameters were measured on 8-10 occasions spanning the intervention. The subjects were randomly divided among hypobaric hypoxia (HYPO n=11) and normoxic (NORM n=12) groups. Apart from doubling EPO concentration 3h after hypoxia there was no increase in any of the measures for either HYPO or NORM groups. The mean change in RCV from PRE to POST for the HYPO group was 2.3% (95% confidence limits = -4.8 to 9.5%) and for the NORM group was -0.2% (-5.7 to 5.3%). The corresponding changes in  $Hb_{mass}$  were 1.0% (-1.3 to 3.3%) for HYPO and -0.3% (-2.6 to 3.1%) for NORM. There was good agreement between blood volume (BV) from EB and CO;  $BV_{EB} = 1.03 BV_{CO} + 142$ ,  $r^2=0.85$ ,  $p<0.0001$ . Overall, evidence from four independent techniques (RCV,  $Hb_{mass}$ , reticulocyte parameters and sTfr) suggests that IHE did not accelerate erythropoiesis despite the increase in serum EPO.

## Introduction

Exposure to high altitude (4000-5500m) is classically associated with an increase in serum erythropoietin (EPO) concentration (38) after as little as 120 min of exposure (30). Chronic exposure to this degree of hypoxia has also been associated with polycythemia, increased hemoglobin concentration, percent reticulocytes and blood volume in South American natives and in sea level residents sojourning to these altitudes (37,42). Modern understanding of EPO biology has clarified that hypoxia initiates EPO gene transcription (31,51), and that hypoxia inducible factor-1 (HIF-1) is the pivotal oxygen-regulated transcription factor responsible for this response pathway (55). Gene expression is up-regulated when hypoxia is present for more than a few minutes (55).

Despite this orthodox and well-established paradigm, the extent of erythropoiesis and benefits for sea level performance of athletes after living and training at moderate altitude (~2200-2500m) for 2-4 wk are controversial (22,50). The degree of erythropoiesis after 2-4 wk living in moderate hypoxia (equivalent to ~2200-2500m) and training near sea level is also contentious (19,34), although this may be due to an inadequate dose of hypoxia in studies that expose their athletes to less than 12 h/d of hypoxia for less than 3 weeks(50). A very time efficient approach of administering hypoxia has been proposed recently as a method to stimulate red blood cell production (11,47,49) as well as  $VO_{2max}$  (by ~5%) and performance (by ~1%) (48). This method, called intermitted hypoxic exposure (IHE), applies severe hypoxia (~4000-5500m) at rest for 1.5 to 5 h/d for 2-3 wk. Exposure periods beyond 2 h at 5450m have previously been demonstrated to yield a 50% increase in serum EPO (30), and even shorter periods (90 min) of exposure to a

simulated altitude of 5500m elicited a 55% increase in serum EPO 3 h after the termination of hypoxia (49). However, most of the IHE studies have inferred an erythropoietic effect from indirect markers such as increased hematocrit, red blood cell count, and hemoglobin concentration (47,49). The mechanism by which IHE increases performance is also contentious since others have observed neither evidence of erythropoiesis nor an increase in  $VO_{2max}$  after severe hypoxia but worthwhile improvements in 3,000m run time (~1.3%) (29). Finally, the results of all IHE studies suffer from a lack of double-blinding.

Collectively, there is sufficient conflicting evidence about the effect and likely mechanisms of resting in severe hypoxia to warrant further investigation. Therefore, the present double-blind, randomized, study was designed to investigate the effects of hypobaric IHE (3 h/d, 5 d/wk, for 4 wk) combined with sea-level training on hematological parameters in well-trained collegiate runners and swimmers. We hypothesized that, in contrast to placebo conditions, IHE would be associated with a significant increase in serum EPO, RCV and  $Hb_{mass}$ .

## Materials and Methods

### *Subjects*

Twenty-seven well-trained athletes (15 swimmers (9 men, 6 women)) and 12 runners (7 men, 5 women) were recruited from high school, collegiate and Masters teams. Two of the male swimmers and two of the male runners completed duplicate tests at baseline, but did not commence the intervention (see study design) leaving a final sample size of  $n = 23$ . Their characteristics are provided in Table 1. All subjects were sea-level residents and trained at least daily for 10 months prior to the study and continued to train throughout the 10 week study, which was conducted in Dallas during May – July, 2003. Each subject provided their voluntary written informed consent to participate in protocols approved by the Institutional Review Boards of the University of Texas Southwestern Medical Center and Presbyterian Hospital of Dallas.

### *Study design*

As part of a multi-centre international collaboration, a variety of measures not reported here were conducted on these subjects; four associated papers will address the effect of IHE on performance, economy, hypoxic ventilatory response, and autonomic function. The overall study design is summarized in Figure 1. After completing duplicate baseline measures for most variables subjects were matched for gender, performance level and training history, and then assigned to either 4 wk of hypobaric hypoxia (HYPO; simulated altitude of 4000-5500m) or normobaric normoxia (NORM; 0-500m) in a randomized, double blind, placebo controlled trial. Carbon monoxide rebreathing was conducted twice before (PRE) and twice after (POST) treatment, whereas Evans blue dye was administered once before and once after hypoxic or placebo intervention because of limited dye availability. Venous blood was drawn eight times

during the 10-wk study (twice before, once per week during and twice after) and analyzed for hemoglobin concentration [Hb], hematocrit (Hct), percent reticulocytes (%Ret), mean cell volume (MCV), mean cell volume of reticulocytes (MCVr), total hemoglobin content of reticulocytes (RetHb = number of reticulocytes x per cell Hb content of reticulocytes), soluble transferrin receptor concentration (sTfr) and erythropoietin concentration (EPO). The samples taken each week during the period of hypoxia or placebo were taken just prior to entering the hypobaric chamber, ~20 hours after the previous hypoxic exposure. Venous blood was also drawn twice (Wk 2, Wk 4) at approximately 3h after chamber exposure and assayed for EPO. Two weeks before, and one week after the intervention, venous blood was assayed for ferritin concentration. During this interval, all subjects received oral liquid iron supplementation (Feo-Sol, 9mg elemental iron·ml<sup>-1</sup>) with the dose (ranging from 5-15 ml, 1-3 times/day) adjusted based on their pre-intervention plasma ferritin concentration.

#### *Hypobaric hypoxia or placebo*

A hypobaric chamber (Perry Baromedical, West Palm Beach, FL, USA), located at the Institute for Exercise and Environmental Medicine, Dallas, was utilized for the experiment and its air refresh rate was calculated to keep the inspired CO<sub>2</sub> fraction < 0.2%. This chamber has three separate locks that can be operated independently and controlled for simulated altitude and rate of ascent/descent. Four chamber runs per day were scheduled to accommodate the living and training schedules of all subjects, divided into two treatment and placebo interventions each day in separate locks. Each chamber exposure, regardless of altitude, included a 10 min ascent and 10 min descent within the 3 hours period. For the NORM group, the first 10 min of exposure involved multiple pressure changes as follows: min 1.5 – 1,800m; min 2.5 – 900m; min 5 –

3,700m; min 6 – 2,500m; min 7 – 3,000m; min 10 – 500m where it remained for the duration of the exposure. United States Air Force standard atmospheric tables were used to derive the barometric pressure for each altitude (59).

### *Hemoglobin mass*

Hb<sub>mass</sub> was estimated using a modified version of a CO-rebreathing technique (10) with a total re-breathing volume of 3.1 L. Following an initial priming dose of 99.9% CO (15 ml for women and 20 ml for men) re-breathed for 10 min, blood was sampled from an antecubital vein via a cannula (20-G, Medex Inc, Carlsbad, CA) into a capped 5 ml glass syringe pre-rinsed with liquid heparin (1000 IU/ml). A second dose (1.25 ml/kg for women and 1.5 ml/kg for men) of 99.9% CO was then re-breathed for 10 min after which a second blood sample was obtained whilst the athlete remained on the re-breathing circuit. When a subject was connected to the re-breathing system, medical-grade O<sub>2</sub> was bled in continuously at a rate equivalent to its consumption; approximately 200-350 ml/min.

Each blood sample was stored on ice until assayed for percent carboxy-hemoglobin (%HbCO) and hemoglobin concentration [Hb] using a diode-array spectrophotometer (OSM3 hemoximeter, Radiometer Medical, Copenhagen). Samples were analysed repeatedly until 5 values for %COHb were obtained within 0.1% of each other, which was usually achieved within 6 replicates. The OSM3 hemoximeter was calibrated according to manufacturer's specifications for Hb concentration (using S2190 Calibrating Solution, Radiometer, Copenhagen) immediately before the PRE and POST data collection periods.

The change in %HbCO ( $\Delta\% \text{HbCO}$  = difference between first and second measures) was used to calculate the  $\text{Hb}_{\text{mass}}$  through which the CO had distributed using the method of Burge and Skinner (10); that is,  $\text{Hb}_{\text{mass}} \text{ (g)} = (\text{Volume CO}_{\text{added STPD}} / 1.34 \text{ ml CO per g Hb}) / \Delta\% \text{HbCO}$ , and where the Volume of  $\text{CO}_{\text{added}}$  is multiplied by 0.978 to correct for the amount of CO remaining in the re-breathing circuit after 10 min (10). The same mercury barometer (Princo, Southampton, PA) and mercury in glass thermometer (Baxter Scientific, Deerfield, IL) was used for all tests to determine the ambient conditions to enable the transformation of the CO gas volume to standard temperature and pressure. The %typical error (TE – see *Statistics* subsection) based on the first two trials for  $n = 27$  was 2.0% (95% CL = 1.6 – 2.7%) for a group mean of 823 g or absolute TE of 17g. When  $\text{Hb}_{\text{mass}}$  was measured over a longer period of time (i.e., from PRE1 to POST1, similar to the time period of measurement for EB derived volumes), the TE was increased to ~3.8% (95% CL 3.0 – 5.5%).

$\text{Hb}_{\text{mass}}$  calculated as described above is not volumetric and therefore does not depend upon determination of [Hb] or Hct (20). However, to allow direct comparison to Evans Blue derived red cell volume (RCV),  $\text{Hb}_{\text{mass}}$  was divided by [Hb] to allow estimation of blood volume via CO ( $\text{BV}_{\text{CO}}$ ). The [Hb] was the mean of the six values determined on the OSM3 hemoximeter for the last blood sample; that is, after 30 min of semi-recumbent rest.

#### *Plasma volume, blood volume and red cell volume*

The plasma volume (PV) of the fasted subjects was determined using the Evans blue dye technique (39). PV was measured subsequent to CO-rebreathing since Evans blue dye interferes with the spectrophotometric measurement of %HbCO (40).

Even though subjects were semi-recumbent throughout the 30 min required to measure  $Hb_{mass}$ , a further 20 min of fully supine rest preceded the PV procedure. An initial blood sample was then taken to provide a plasma blank and for determination of Hct and other haematological indices. Immediately thereafter, ~2.5 ml of Evans blue was injected intravenously and blood re-sampled ~10, 20 and 30 min later. The exact dye volume was determined as initial minus final syringe mass determined on a 0.1 mg resolution balance (Mettler-Toledo GmbH AE100, Columbus, OH). After centrifugation, PV was calculated from time zero extrapolation of the regression derived from the 3 post-injection absorbances at 620 and 740 nm (Hewlett Packard model 8452A diode array spectrophotometer). Hct was measured in duplicate with a microcapillary centrifuge and blood volume via Evans blue ( $BV_{EB}$ ) was calculated as  $PV/(1-Hct)$ , using a 0.96 correction for plasma trapping associated with a spun Hct and 0.92 for peripheral sampling. RCV was then calculated as  $BV - PV$ . Optical density (OD) of the batch of Evans blue dye used in this experiment was measured directly at 620 nm from diluted (1,000 times) Evans blue dye obtained from two different ampoules as the average of 3 repetitions from each sample. The mean OD (0.39625) x the dilution ratio was then multiplied by the specific extinction of Evans Blue dye to determine the actual initial dye concentration. The %TE using the PRE vs POST data for PV, RCV and  $BV_{EB}$  were 7.2 (95% CL = 5.8 - 10.8%), 6.7 (95% CL = 5.3 - 10.0%) and 6.0% (95% CL = 4.7 - 8.8%), respectively.

#### *Hematological indices*

Erythrocyte and reticulocyte parameters were analysed using an ADVIA 120 Hematology Analyzer (Bayer Diagnostics, Tarrytown NY) which performs flow cytometric measurements (8).

All analyses were completed within 8 h of collection. The ADVIA was calibrated against appropriate reference materials, and controlled daily using Bayer ADVIA *TESTpoint* Haematology Low, Normal and High controls and Bayer ADVIA *TESTpoint* Reticulocyte Low and High controls. The %TE (and 95% CLs) from the two PRE measures for the parameters reported in our results were as follows: Hct 4.0% (3.2-5.9%), [Hb] 3.7% (2.9-5.3%), %Retic 19.5% (16.3-31.8%), MCV 0.5% (0.4-0.7%); MCVr 1.2% (0.9-1.7%); and RetHb 21.6% (19.0-33.4%).

All serums were separated and aliquoted into cryotubes, stored at  $-20^{\circ}\text{C}$  or  $-80^{\circ}\text{C}$ , packed on dry-ice, and then shipped to Australia for analysis. The EPO and sTfr concentrations were determined using an automated solid-phase chemiluminescent immunoassay (Diagnostic Products Corporation, Los Angeles, CA, USA) and an automated immunonephelometric assay (Dade Behring, Germany), respectively. The automated immunoassays for EPO and sTfr were controlled using three and two levels of controls, respectively. We have previously reported on the calibration procedures as well as the within- and between-assay coefficients of variation (CV) in our hands (56). Ferritin concentration was measured using a Hitachi 911 Biochemistry Analyzer (Roche Diagnostics, Rotkreuz, Switzerland) and we have previously attained a mean CV during a 6 month study of 5.5% over the range of 15-500  $\text{ng}\cdot\text{ml}^{-1}$  (56).

### *Statistics*

All data are expressed as mean  $\pm$  SD, with most analyses completed using Statistica (version 6.0, Tulsa, Oklahoma). For blood volumes and  $\text{Hb}_{\text{mass}}$ , repeated measures ANOVA was used to compare Groups (HYPO vs NORM) by Time (duplicate or single measures PRE and POST).

Repeated measures ANOVA for Group by Time was also used for the blood and serum parameters that were measured on eight or ten occasions. After significant main effects or interactions, Tukey post-hoc tests were used to identify difference between cell means. Linear regression was used to examine relationships between variables, and typical error ( $TE = SD$  of difference scores /  $\sqrt{2}$ ) was used to quantify reliability expressed in both absolute units or as a percentage of the mean. Slopes and intercepts were compared with the line of identity using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California). Two of the 23 subjects (both from the NORM group) were unable to complete the POST2 measure of  $Hb_{mass}$ . In order to avoid casewise deletion in the associated ANOVAs, substitution of the mean of their other three values was used for their fourth value. Mean substitution was not used for TE calculations.

## Results

### *Hb<sub>mass</sub>*

Hb<sub>mass</sub> did not increase significantly in response to hypoxia or placebo, Figure 2. When the data of the duplicate PRE and POST trials were averaged, the means ( $\pm$ SD) for the HYPO group were 789 ( $\pm$ 188) and 794 ( $\pm$ 181) g before and after hypoxia, respectively. The corresponding values for the NORM group were 818 ( $\pm$ 188) and 819 ( $\pm$ 181) g. The overall mean change in Hb<sub>mass</sub> for the HYPO group was 1.0% (95% CL = -1.3 to 3.3%) and for the NORM group was -0.3% (95% CL = -2.6 to 3.1%) when calculated from the individual percent changes from PRE to POST.

### *PV, RCV and BV<sub>EB</sub>*

PV (Figure 2) as well as RCV and BV<sub>EB</sub> did not change significantly with hypoxia or placebo with the respective latter two Group by Time ANOVA results of  $F_{(1,21)} = 0.04$ ,  $p = 0.84$ ; and  $F_{(1,21)} = 0.61$ ,  $p = 0.44$ . The individual and mean data for RCV are illustrated in Figure 2.

Calculated from the individual percent changes the overall mean change in RCV from PRE1 to POST1 for the HYPO group was 2.3% (95% CL = -4.8 to 9.5%) and for the NORM group was -0.2% (95% CL = -5.7 to 5.3%).

The change in RCV was not significantly correlated with the change in Hb<sub>mass</sub> whether expressed in absolute terms ( $r^2 = 0.10$ ,  $p = 0.14$ ) or as a percent ( $r^2 = 0.08$ ,  $p = 0.20$ ). The mean change in RCV after intermittent hypoxia or placebo for the pooled HYPO and NORM groups was 1.0% (95% CL = -3.3 to 6.1%) and that for Hb<sub>mass</sub> was 0.6% (95% CL = -1.1 to 2.3%).

### *Blood volume comparison*

For the pooled data of both HYPO and NORM groups before and after intermittent hypoxia or placebo the mean  $BV_{EB}$  was  $5696 \pm 1043$  ml and  $BV_{CO}$  was  $5738 \pm 995$  ml. The Pearson correlation between methods was statistically significant ( $r^2 = 0.85$ ,  $p < 0.0001$ ; Figure 3). Both the slope ( $p = 0.90$ ) and the intercept ( $p = 0.96$ ) of this regression were not different from those of the line of identity. The TE 95% confidence intervals for blood volume measured using these two independent techniques were overlapping:  $BV_{CO} = 3.1 - 5.8\%$ ;  $BV_{EB} = 4.7 - 8.8\%$ .

### *Hematological indices*

Serum EPO concentration was significantly increased in the HYPO group 3 h following the hypoxic exposure at both Wk2 and Wk4 compared with their own PRE1 and PRE2 values but not compared with the temporally matched values of the NORM group (Group x Time interaction;  $F_{(9,162)} = 10.3$ ,  $p < 0.0001$ ), Table 2. The increase in EPO 3 h after hypoxia in the HYPO group at Wk 4 was not different to that at Wk 2 ( $p = 0.19$ ). There was a significant decline in sTfr for the HYPO group but not the NORM group ( $F_{(9, 62)} = 2.8$ ,  $p < 0.01$ ). The sTfr concentrations at Wk3, Wk4, Wk4+3h and POST2 were all lower than the PRE1 and PRE2 values for the HYPO group. There were no significant changes within or between the HYPO and NORM groups for [Hb], Hct, %Ret, RetHb, MCV or MCVr (Table 2), and the p-values for the Group by Time interactions were 0.38, 0.85, 0.54, 0.44, 0.80 and 0.31, respectively. Serum ferritin tended to increase in the HYPO group and decline in the NORM group from PRE to POST (Table 2), but the Group by Time interaction failed to reach conventional significance ( $F_{(1,21)} = 3.5$ ,  $p = 0.08$ ).

## Discussion

The major finding of this study is that 4 wk of IHE (simulated altitude of 4000-5500m for 3 h/d, 5 d/wk) was insufficient to increase RCV or  $Hb_{mass}$ . This finding was supported by the absence of any evidence of accelerated erythropoiesis from a comprehensive assessment of indirect markers such as soluble transferrin receptor or red cell indices derived from flow cytometry. Together, these findings suggest that this dose of hypoxia is inadequate to stimulate erythropoiesis, despite significant increases (doubling) in serum EPO 3 h after each hypoxic treatment. The second main outcome is that there was good agreement between the blood volumes determined with Evans blue and CO-rebreathing. This concordance is consistent with reports that CO is minimally off-loaded during 10 min of rebreathing (7), and provides strong evidence that both techniques are appropriate to use for the evaluation of the red cell compartment after exposure to hypobaric hypoxia given an expected change greater than the typical error of the technique used.

### *Lack of increased erythropoiesis*

The degree and extent of hypoxia to increase serum EPO is quite well characterized and there is no doubt that exposure to just a few hours of adequate hypoxia increases production of EPO (1,14,30). For example, eighty-four min at 4000m was sufficient to significantly increase serum EPO (14), ninety minutes at 5500m increased serum EPO by 55% (49) and, after 3 h of continuous hypoxia, EPO was 120% of the pre-exposure concentration. Likewise 2 h at 5450m yielded a 50% increase in serum EPO (30) and, in our HYPO group, there was a doubling of serum EPO 3 h after both the fourth and nineteenth exposures at 4500 and 5500m, respectively. This robust increase in EPO in response to repeated exposure to simulated high altitude (4000-5500m) for 3 h/d for 4 wk has also been reported when IHE is administered to triathletes (2). For chronic exposure to terrestrial altitude, it has been confirmed that an altitude of ~2200m is

necessary to reach the steep part of the oxyhemoglobin dissociation curve when arterial oxygen tension falls below 65 mm Hg (62). More recent work makes it clear that there is substantial between subject variation in the threshold altitude but for most people it appears to be between 2100 and 2500m (17). With a minimum altitude of 4000m and duration of 3 h in the current study of IHE, it is clear that we exceeded the dose of hypoxia required to increase serum EPO.

It has been previously assumed that a sustained increase in serum EPO will lead to an obligatory increase in the total RCV and hence  $Hb_{mass}$  (15,26). We used two independent methods to measure the red blood cell compartment and neither yielded a significant mean change with the HYPO group. One possible explanation is that the measurement techniques were too insensitive to measure an important change. But the mean changes in the HYPO group were just 2.3% and 1.0% for RCV and  $Hb_{mass}$ , respectively, which corresponds to 46 ml of red blood cells and 8 g of Hb relative to the PRE values for that group. The constancy of the red blood cell compartment in the face of IHE is supported by the lack of change in [Hb], Hct, %Ret, RetHb and  $MCV_r$ , and was likely not due to inadequate iron (57) since serum ferritin tended to increase, not decrease, in the HYPO group and both groups were supplemented with iron throughout the study. Finally, sTfr did not increase in the HYPO group and sTfr is considered as a prime and sensitive marker of the erythroid mass (25). Therefore evidence from four independent techniques (RCV,  $Hb_{mass}$ , reticulocyte parameters and sTfr) suggest that IHE did not accelerate erythropoiesis despite the transient increases in serum EPO.

An alternate explanation is that despite the doubling of serum EPO, the 21 h spent in normoxia each day, as well as the weekend spent in normoxia, were sufficient to counter the transient

increases induced by IHE. Serum EPO clearance and total area under the curve warrant consideration as possible mechanisms, though counterbalancing pathways for inhibition of the hypoxia response pathway also should be considered. Both the rapid destruction of hypoxia-inducible factor 1alpha HIF-1  $\alpha$  (27) (60) and a marked decrease in red cell survival time (42), termed 'neocytolysis' (3,43), may compromise the ability of short duration hypoxia to increase the red cell mass.

The total "dose" of hypoxia required to increase RCV and Hb<sub>mass</sub> is uncertain (19,27,33,34). For example, it was previously reported that 31 d at 2690m did not increase the Hb<sub>mass</sub> of a small number of cyclists (18), though some of them were ill during the final week of their altitude sojourn which may have inhibited their EPO response. In contrast, 4 wk at 20-22 h/d at 2500m was sufficient to increase RCV of a large number of runners (32) and a cross sectional study has observed a higher Hb<sub>mass</sub> of cyclists who are life-long residents at 2600m (54). More recently, accelerated erythropoiesis has been confirmed in elite athletes at moderate altitudes (16,23,54,61). Thus despite rare exceptions (18), the accumulated evidence from several research groups has confirmed that sufficient durations of moderate altitude exposure for nearly 24 h/d increases the red cell mass even in elite athletes.

But what about more intermittent exposures? It has been shown that 12-16 h/d of normobaric hypoxia for 3 (9) or 4 wk (50) closely replicates the results observed in the field studies with an increase in both Hb<sub>mass</sub> and VO<sub>2max</sub>. In contrast, 8-10 h/d of normobaric hypoxia (2,500-3,000m) for 10-21 d did not increase in Hb<sub>mass</sub> or VO<sub>2max</sub> (5). We speculate that this "dose" of intermittent

moderate hypoxia exposure, in which the duration of time spent in normoxia exceeds that spent in hypoxia, may be insufficient to initiate and sustain a robust erythropoietic response (35).

*Comparison with other studies using short duration but severe hypoxic exposure*

Our double-blind protocol of 5:5min of hypoxia (10%):normoxia for 70 min 5 times/wk recently failed to find any increase in erythropoiesis based on reticulocyte parameters or sTfr (28), which may have been due to an inadequate duration of hypoxia (1,14,30). On the other hand, it has been reported that 180-300 min/d of IHE (4000-5500m) for 9-17 d was sufficient to increase Hct, [Hb] and red blood cell count by ~10% (11) and double the percentage of reticulocytes (47). Similar increases were observed after just 90 min or IHE 3 times/wk for 3 wk (49). Plasma volume loss could explain most of these observations and additional exercise can stimulate reticulocytes (4). A recent study by this Spanish research group (2) failed to demonstrate an increase in reticulocytes using the identical model of IHE as employed in the present investigation; instead of using the imprecise manual method of reticulocyte counting (52,58), for the first time they used an automated flow-cytometer analyzer for this measurement. In addition, hypobaric hypoxia can stimulate the release of immature red cell forms from the bone marrow thus increasing reticulocytes without actually accelerating erythropoiesis (21). Interestingly, the consistent rise in EPO in the 4<sup>th</sup> week compared to the 2<sup>nd</sup> week of our study is similar to that observed in Chilean miners or European sojourners who are exposed to repetitive severe hypobaric hypoxia for many years (21) but in contrast to the widely reported gradual decrease in EPO during sustained high altitude residence (21,32,44). We suggest that finding is one more marker of the failure to increase blood oxygen content during chronic intermittent hypobaric hypoxia and argues against a vigorous acclimatization response.

Failure to use a control group exposed to a placebo altitude was a limitation of most (11,47,49), but not all (2) the previous IHE studies. The current study circumvented this shortcoming and is only the second double-blind investigation to have administered hypoxia to athletes; the first was Julian and coworkers (28). The additional benefit of double baseline measures of the haematological variables and a control group against which to assess them allows for confidence in our results.

Overall, it appears that in order to accelerate erythropoiesis with IHE, not only the level of EPO increase induced by the hypoxic exposure has to be considered, but also other factors such as the duration of the normoxic conditions after each IHE exposure, the training history of the athletes, and possibly the extent of training-induced neocytolysis subsequent to an increase in  $Hb_{\text{mass}}$ . These factors may also play integrated, non-linear roles in the increase of the erythroid mass after intermittent hypoxia as suggested elsewhere (46).

#### *Hemoglobin mass and red cell volume – agreement of methods*

We attained good agreement between the BV assessed via RCV and that estimated from CO-rebreathing. One of the prime criticisms of the CO-rebreathing method has been that it overestimates the  $Hb_{\text{mass}}$  because it is distributed beyond the circulation to non-blood iron porphyrin molecules such as in muscle and liver (24,41,53). However, CO is produced endogenously (13) and furthermore in the presence of normal or high inspired  $O_2$ , as used previously (10,18), oxygen binds preferentially to myoglobin even in the presence of CO (12,36,36). There is also evidence that CO loaded on to red blood cells via the lungs is not redistributed between

erythrocytes during circulation (6). Recently it has been demonstrated that with 14 min of supra-systolic occlusion at rest the deoxy-myoglobin signal is not attenuated with a pre-load of 20% COHb (45). Richardson and colleagues (45) conclude that these data support Burge and Skinner's previous contention (10) that it is not necessary to correct measures of  $Hb_{mass}$  for loss of CO to myoglobin.

In the current experiment, the two methods agreed closely and Evans blue tended to marginally overestimate the BV (by 3%) compared with that estimated from CO-rebreathing. The 3% 'difference' is likely trivial since the slope of the line for the regression between the two methods was not different from the line of identity. Overall, the data from the current experiment demonstrate that, when carefully performed, both methods are suitable to measure BV and associated variables in athletes undergoing hypobaric hypoxia; although averaged across many studies  $Hb_{mass}$  has better reliability than RCV derived from Evans blue dye (20), and is the preferred method when changes are anticipated to be small.

### *Conclusion*

Using a double-blind protocol, four weeks of hypobaric hypoxia equivalent to 4000-5500m altitude for 3 h/d, 5 d/wk was insufficient to increase RCV or  $Hb_{mass}$ . Evidence from four independent techniques (RCV,  $Hb_{mass}$ , reticulocyte parameters and sTfr) confirm that IHE did not accelerate erythropoiesis despite the transient increase in serum EPO. The dissociation between an increase in serum EPO without an increase of red blood cells alludes to the requirement of a larger and more sustained, critical dose of EPO to yield a substantial effect on the red cell compartment.

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## Figure Legends

**Figure 1:** Testing schedule and simulated altitude exposure of the hypobaric hypoxia (HYPO, n = 12) and normoxic (NORM, n = 11) groups with treatment administered in a double-blind placebo fashion. The HYPO group were exposed to 4000-5500m 3h/d for 5 d/wk whereas the NORM group were exposed to 0-500m of simulated altitude.

**Figure 2:** Individual and mean hemoglobin mass ( $Hb_{mass}$ ; top panels) and red cell volume (RCV; bottom panels) before and after 4wk of intermittent hypoxia or placebo (3 h/d, 5 d/wk).

**Figure 3:** The correlation between blood volume measured via Evans blue dye and CO-rebreathing. Pooled data from 23 subjects before (PRE) and after (POST) 4 wk of intermittent hypoxia or placebo.

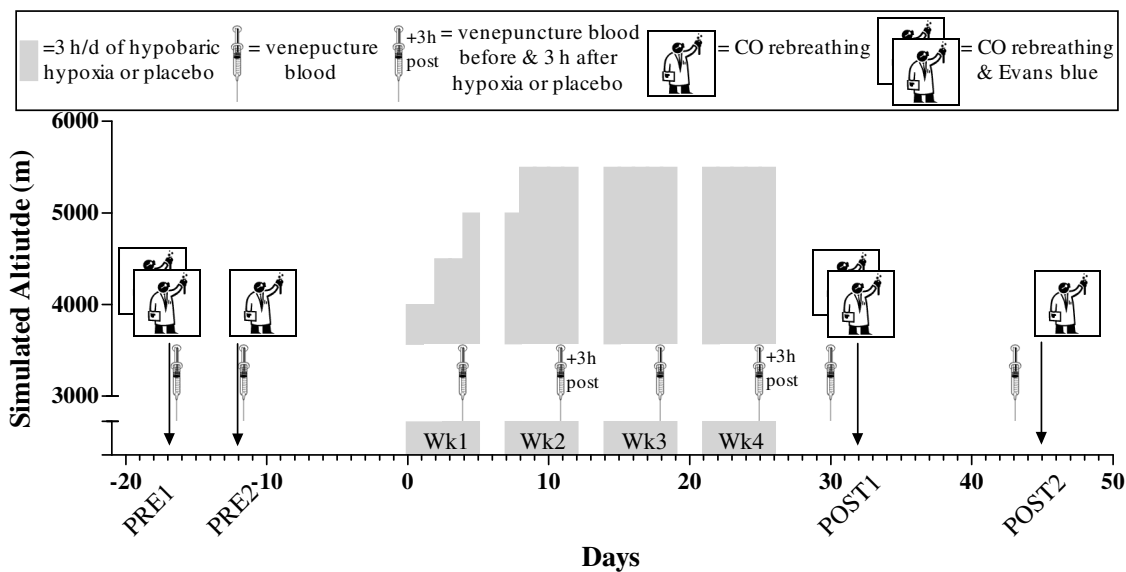


FIGURE 1

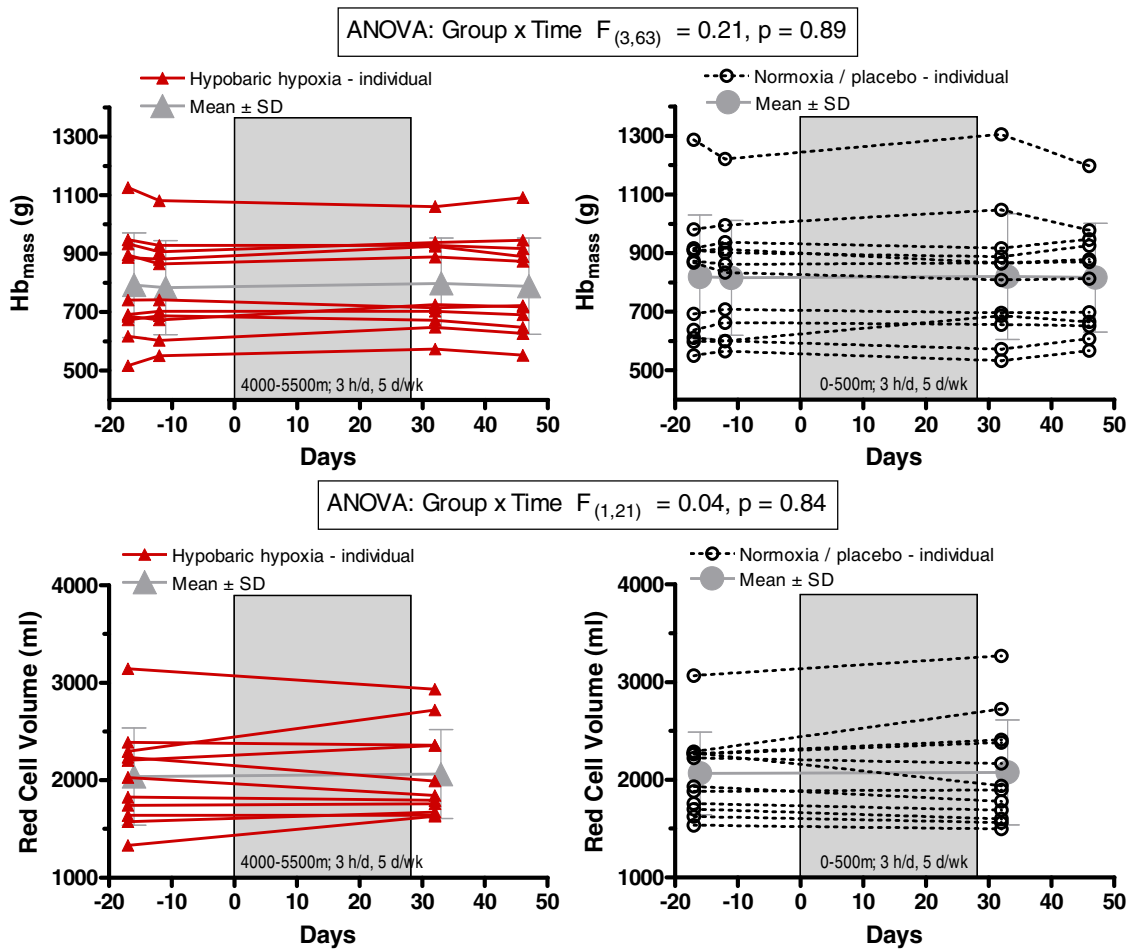


FIGURE 2

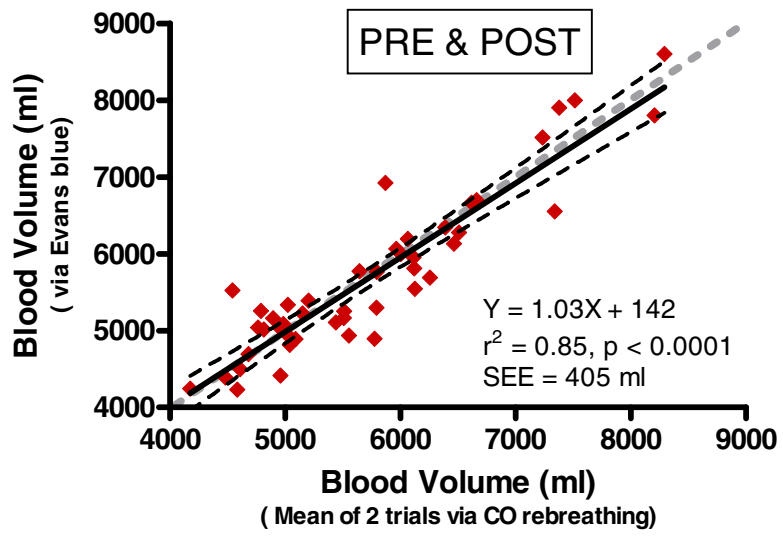


FIGURE 3

## Tables

**Table 1.** Subject characteristics. Values are mean  $\pm$  SD and range in parentheses.

<b>Group</b>	<b>n</b>	<b>Age</b> <b>(yr)</b>	<b>Height</b> <b>(cm)</b>	<b>Mass</b> <b>(kg)</b>	<b>Blood Volume<sup>a</sup></b> <b>(ml/kg)</b>
HYP0	11 (5 men, 6 women)	22.6 $\pm$ 7.0 (16.3-35.4)	171.6 $\pm$ 8.2 (160.0-185.5)	66.6 $\pm$ 12.15 (48.3-90.8)	84.8 $\pm$ 11.7 (72.1-106.9)
NORM	12 (6 men, 6 women)	23.2 $\pm$ 9.3 (16.2-48.3)	174.9 $\pm$ 9.9 (162.6-193.0)	67.3 $\pm$ 12.05 (51.3-96.8)	85.0 $\pm$ 8.7 (71.5-99.2)
Swimmers	13 (7 men, 6 women)	20.6 $\pm$ 8.8	178.0 $\pm$ 8.4	73.0 $\pm$ 11.7	81.8 $\pm$ 8.5
Runners	10 (5 men, 5 women)	25.8 $\pm$ 6.2	167.2 $\pm$ 5.9	59.1 $\pm$ 6.2	89.0 $\pm$ 10.8
Males	12	22.5 $\pm$ 8.9	178.1 $\pm$ 9.1	70.3 $\pm$ 12.0	88.1 $\pm$ 10.4
Females	11	23.2 $\pm$ 7.4	168.1 $\pm$ 5.6	63.3 $\pm$ 11.0	81.5 $\pm$ 8.7

HYP0 = Hypobaric Hypoxia (4000-5500m)

NORM = Normoxia (0-500m)

<sup>a</sup> = Blood volume estimated from Evans blue prior to hypoxia or placebo.

**Table 2:** Hematological response to 4 weeks of intermittent hypoxia or placebo. Values are mean and (SD).

Variable	Group	PRE1	PRE2	Wk1	Wk2	Wk2 +3 h	Wk3	Wk4	Wk4 + 3h	POST1	POST2
<b>EPO</b> (IU/L)	HYPO n=8	15.7 (9.8)	15.4 (8.0)	11.0 (6.6)	12.0 (8.0)	*36.4 (15.0)	8.7 (4.1)	11.0 (6.0)	*31.3 (5.9)	11.4 (5.7)	10.6 (2.7)
	NORM n=12	12.0 (4.6)	12.5 (5.3)	12.6 (4.0)	11.1 (4.7)	15.1 (5.8)	11.0 (4.8)	13.0 (8.7)	12.4 (20.0)	12.4 (4.4)	11.4 (4.0)
<b>sTfr</b> (ng/ml)	HYPO n=8	1.7 (.3)	1.7 (.5)	1.6 (.3)	1.6 (.4)	1.6 (.4)	*1.4 (.5)	*1.5 (.3)	*1.5 (.3)	1.5 (.3)	*1.5 (.3)
	NORM n=12	1.6 (.5)	1.6 (.3)	1.5 (.4)	1.6 (.3)	1.6 (.3)	1.6 (.4)	1.5 (.3)	1.6 (.3)	1.6 (.3)	1.6 (.3)
<b>[Hb]</b> (g/dL)	HYPO n = 10	14.4 (1.4)	14.4 (1.1)	14.4 (1.0)	14.6 (1.2)	-	14.3 (1.0)	14.4 (0.9)	-	14.7 (0.8)	14.6 (0.9)
	NORM n = 12	14.4 (1.4)	14.3 (1.0)	14.1 (1.1)	14.2 (1.1)	-	14.0 (0.9)	14.1 (1.0)	-	14.1 (1.0)	14.2 (0.9)
<b>Hct</b> (%)	HYPO n = 10	42.9 (3.3)	43.4 (2.5)	43.2 (2.8)	44.1 (3.4)	-	43.3 (3.2)	43.5 (2.7)	-	43.6 (2.2)	43.8 (2.8)
	NORM n = 12	43.1 (4.1)	43.2 (2.5)	42.2 (2.6)	42.9 (3.0)	-	42.3 (2.1)	42.3 (2.8)	-	42.7 (3.1)	42.6 (2.4)
<b>%Ret</b> (%)	HYPO n = 10	1.7 (.4)	1.8 (.4)	1.8 (.4)	1.8 (.5)	-	1.7 (.4)	1.7 (.4)	-	1.6 (.4)	1.6 (.3)
	NORM n = 12	1.9 (.4)	1.7 (.3)	1.6 (.3)	1.8 (.5)	-	1.7 (.3)	1.7 (.3)	-	1.7 (.5)	1.6 (.3)
<b>RetHb</b> (g/L)	HYPO n = 10	2.5 (.6)	2.6 (.6)	2.7 (.5)	2.8 (.6)	-	2.7 (.7)	2.7 (.5)	-	2.7 (.6)	2.5 (.6)
	NORM n = 12	2.8 (.5)	2.5 (.4)	2.4 (.4)	2.6 (.6)	-	2.6 (.5)	2.7 (.5)	-	2.8 (.8)	2.5 (.4)
<b>MCV</b> (fL)	HYPO n = 10	88.7 (4.4)	88.8 (4.3)	88.3 (4.2)	88.8 (4.3)	-	88.5 (4.4)	88.6 (4.1)	-	88.6 (4.4)	89.4 (3.7)
	NORM n = 12	88.7 (3.8)	88.8 (3.7)	88.8 (3.5)	89.3 (3.4)	-	88.9 (3.7)	88.5 (4.1)	-	88.6 (4.1)	89.7 (4.4)
<b>MCVr</b> (fL)	HYPO n = 10	107.8 (4.3)	107.5 (4.1)	107.4 (3.8)	107.7 (4.6)	-	106.9 (4.5)	107.6 (4.2)	-	107.9 (3.8)	108.0 (2.7)
	NORM n = 12	106.7 (3.4)	107.0 (3.3)	107.6 (3.8)	107.5 (3.6)	-	106.9 (3.8)	107.1 (3.3)	-	106.3 (4.0)	107.6 (3.8)
<b>Ferritin</b> (ng/ml)	HYPO n = 11	79.7 (48.6)	-	-	-	-	-	-	-	87.3 (37.1)	-
	NORM n = 12	94.9 (88.7)	-	-	-	-	-	-	-	73.1 (54.1)	-

EPO = serum erythropoietin concentration, sTfr = soluble transferrin receptor concentration, [Hb] = hemoglobin concentration, Hct = hematocrit, %Ret = percentage of reticulocytes, RetHb = total reticulocyte hemoglobin (number of reticulocytes x per cell hemoglobin content of reticulocytes), MCV = mean cell volume, MCVr = mean cell volume of reticulocytes.

Wk2+3h and Wk4+3h denote sampling time three h after a three-hour exposure to hypobaric hypoxia or placebo.

\* = significantly different from both PRE1 and PRE2.

