TITLE: Temporal changes in physiology and haematology in response to high- and micro-doses of recombinant human erythropoietin

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ABSTRACT

There is evidence to suggest athletes have adopted recombinant human erythropoietin (rHuEPO) dosing regimens that diminish the likelihood they will be caught by direct detection techniques. However, the temporal response in physiology, performance and athlete biological passport (ABP) parameters to such regimens is not clearly understood. Participants were assigned to a high dose only group (HIGH, n = 8, six rHuEPO doses of 250 IU/kg over two weeks), a combined high micro-dose group (COMB, n=8, high dose plus nine rHuEPO micro-doses over a further three weeks), or one of two placebo control groups who received saline in the same pattern as the HIGH (HIGH-PLACEBO, n = 4) or COMB (COMB-PLACEBO, n = 4) groups. Temporal changes in physiology and performance were tracked by graded exercise test (GXT) and haemoglobin mass assessment at baseline, after high dose, after micro-dose (COMB and COMB-PLACEBO only) and after a four week washout. Venous blood samples were collected throughout the baseline, rHuEPO administration and washout periods to determine the haematological and ABP response to each dosing regimen. Physiological adaptations induced by a two week rHuEPO high dose were maintained by rHuEPO micro-dosing for at least three weeks. However, all participants administered rHuEPO registered at least one suspicious ABP value during the administration or washout periods. These results indicate there is sufficient sensitivity in the ABP to detect use of high rHuEPO doping regimens in athletic populations and they provide important empirical examples for use by anti-doping experts.

KEYWORDS: Doping; micro-dosing; performance; ABP; EPO

INTRODUCTION

Recombinant human erythropoietin (rHuEPO) is a pharmaceutical exogenous form of the glycoprotein erythropoietin, which is released by the kidneys and regulates the production of red blood cells. In healthy and athletic populations, administration of rHuEPO suppresses apoptosis of the erythroid progenitors[1] and thus results in an increase in haemoglobin concentration ([Hb]) and total haemoglobin mass (Hb_{MASS}).[2,3] These haematological adaptations confer improvements in the oxygen carrying capacity of the blood and subsequently increase maximal oxygen uptake (VO_{2MAX}),[2-8] which ultimately improves endurance exercise performance.[2,6,8] Importantly, rHuEPO and other erythropoiesis stimulating agents (ESA) are classified as prohibited substances by the World Anti-Doping Agency (WADA)[9] and as such are banned in and out of competition by various sports governing bodies including the International Olympic Committee.

To negate athlete abuse of rHuEPO, international sports governing bodies, anti-doping authorities and researchers have developed, tested and implemented a number of detection or deterrent methods with varying success. Arguably the most successful of these methods is the Athlete Biological Passport (ABP), which was developed following a series of research studies in the early 2000's.[3,10-14] The ABP relies on serial monitoring of an individual athlete's blood, in particular the haematological parameters [Hb], reticulocyte percentage (Ret%) and the OFF-score, which is derived from an algorithm combing [Hb] and Ret%.[10] Introduction of the ABP by the International Cycling Union (UCI) in 2008 led directly to a number of anti-doping prosecutions, anti-doping related competition bans and evidence of a reduction in rHuEPO abuse.[15,16] Additionally, Zorzoli [15] indicated further anti-doping bans were issued following targeted urine testing of athletes with suspicious ABP results.

Despite the apparent success of some methods in deterring rHuEPO abuse, some researchers suggest athletes have modified their doping regimens to negate detection techniques.[10,17] Specifically, anti-doping experts believe nefarious athletes and support personnel use rHuEPO micro-doses to circumvent both direct and indirect detection methods. Evidence suggests serial micro-dosing provides sufficient erythropoietic stimulus to increase Hb_{MASS} by up to 10%, [18] and is effective at maintaining elevations in [Hb] induced by administration of a two week high rHuEPO dose.[17] While advances in testing techniques have improved the effective window of direct detection methods, recent research indicates the sensitivity of current urine detection techniques is reduced to as low as 41.6% at 20 hours after a single micro-dose.[19] However, there is currently no published data detailing the temporal changes in exercise performance, physiology and parameters of the ABP to micro-dosing subsequent to a high dose administration of rHuEPO. Such information would be of assistance to experts trying to interpret individual ABP profiles.[20,21]

Therefore, the aim of this project was to quantify the temporal changes in physiology, performance and haematology throughout two different regimens of rHuEPO administration and a subsequent four-week washout. Specifically, the project examined the effects of a combined high-low rHuEPO administration period to establish if rHuEPO micro-dosing is sufficient to maintain changes evoked by priming high rHuEPO doses. A secondary aim was to test the sensitivity of the ABP, in particular the OFF-score model, to determine if rHuEPO micro-dosing masks a preceding period of high doses of rHuEPO.

METHODS

Participants

Twenty-four healthy recreational athletes (19 men, 5 women, Table 1) volunteered to participate in this project. No participant was a member of a National or State sporting squad or a scholarship holder at the Australian (AIS) or South Australian Institute of Sport (SASI). All participants underwent medical screening and signed a statement of informed consent before commencing the study. Experimental procedures were reviewed by an appropriate medical professional and approved by the AIS Ethics Committee.

Experimental design

The study used a randomised double-blinded placebo-control design whereby participants were randomly assigned to one of four groups and both researchers and participants were blind to the substance administered to each participant. Participants in group one (HIGH) were administered a subcutaneous high dose of 250 IU/kg of epoietin-beta rHuEPO (Neorecormon, Roche Products Pty Ltd, USA) three times a week for two weeks followed by a four week washout. Group two (COMB) participants received a combination of the two week high dose followed by an additional three week micro-dose period during which they were administered a 10 IU/kg dose of epoietin-beta rHuEPO three times per week. The final two epoietin-beta rHuEPO doses of four participants from the COMB group were titrated to 15 IU/kg. A further two groups acted as placebo controls and were administered equivalent doses of saline across either the same time frame and dosing regimen as the HIGH group (HIGH-PLACEBO) or the COMB group (COMB-PLACEBO). All participants also received a single 100 mg dose of intravenous iron (Venofer, American Regent, Shirley NY, USA) before the high dose administration period. In accordance with the protocol approved by the ethics committee, rHuEPO was replaced with saline for the final two injections for two participants of the COMB group because their haematocrit (Hct) exceeded 0.55. No other injections were missed or substituted.

The testing and rHuEPO administration schedule is presented in detail in Figure 1. Participants in the HIGH and HIGH-PLACEBO groups completed a graded exercise test

(GXT) before rHuEPO administration, immediately post administration (post 3) and after a four week washout (post 30). The participants in the COMB and COMB-PLACEBO groups completed an additional GXT between the high and micro-dose periods (day 13). HIGH and HIGH-PLACEBO participant's total haemoglobin mass was measured in duplicate before rHuEPO administration, and then singly thereafter immediately after administration (post 3) and weekly throughout a four week washout (post day 7, 23 and 30), with an additional measure between high and micro-dose administration periods (day 13) for the COMB and COMB-PLACEBO participants. Blood samples were collected at multiple time points throughout the respective rHuEPO administration and washout periods (HIGH and HIGH-PLC: Pre 10, Pre 3, Post 3, Post 10, Post 17, Post 23 and Post 30; COMB and COMB-PLC: Pre 10, Pre 3, Day 13, Day 22, Day 29, Post 3, Post 10, Post 17, Post 23 and Post 30).

Graded exercise test

Participants completed a GXT to exhaustion on a customised dynamically calibrated cycle ergometer (SASI, Adelaide, Australia) or a customised motorised treadmill (SASI, Adelaide, Australia) in the mode of exercise in which they most frequently trained (cycling n = 11, running = 13). The cycling test began with one minute at 100 W for males and 50 W for females with power output increased by 25 W every minute thereafter until exhaustion. For females the treadmill test began with one minute at 7km·h^{-1} and 1% gradient. Speed increased by 1 km·h^{-1} at the end of every minute until the treadmill reached 11 km·h^{-1} after which gradient increased by 2% per minute until exhaustion. Males completed the same testing protocol however the starting speed was set at 9 km·h^{-1}.

Throughout the GXT, oxygen uptake (VO_2) was measured using an open circuit metabolic system which incorporated a low resistance respiratory valve (Hans Rudolph 2700, Kansas City, MO, USA) with a large turbine volume transducer (PK Morgan, Chatham, Kent, UK) attached to the inspiratory port. The O_2 and CO_2 concentrations of dried expirate were continually monitored by S.3AI O_2 (AEI Technologies Inc., Pittsburgh, PA, USA) and CD.3A CO_2 (AEI Technologies Inc., Pittsburgh, PA, USA) analysers, respectively, which were calibrated before, during (where possible) and after each test using three alpha grade gravimetric reference gases (The Commonwealth Industrial Gases Limited, Adelaide, Australia). The volume transducer was calibrated using a Tissot verified automated three litre pulsatile pump. VO_2 was calculated using the Geppert and Zuntz [22] transformation and the highest 60 second measurement of VO_2 was determined as peak oxygen uptake (VO_{2PEAK}). Time to exhaustion (TTE) was recorded as the time of volitional exhaustion to the nearest whole second. Upon exhaustion, a blood sample was collected from a fingertip capillary vein and immediately assessed for peak blood lactate concentration ([La$^-$]) using an automated lactate analyser (Lactate Pro LT 1710, Arkray Inc, Kyoto, Japan). Additionally, participants

rated their perceived exertion (RPE) using the 15 point Borg scale [23] and peak heart rate was assessed using short range heart rate telemetry (Polar Electro, Kempele, Finland).

Haemoglobin mass estimation

Total haemoglobin mass (Hb_{MASS}) was assessed using the optimised carbon monoxide rebreathing technique as described previously by Schmidt and Prommer [24] Briefly, participants were connected to a spirometer that was used to deliver a bolus of carbon monoxide (~0.8 and ~1 ml/kg body mass for females and males respectively) mixed with four litres of oxygen which was rebreathed from a gas reservoir for two minutes. Change in the percent carboxyhaemoglobin content of arterialised capillary blood (measured with at least four replicates per sample on an CO-oximeter (OSM3, Radiometer, Copenhagen, Denmark) from baseline to six and eight minutes post CO administration, residual CO left in the gas reservoir after rebreathing and end tidal CO at seven minutes post CO administration were used to calculate haemoglobin mass according to the method detailed by Schmidt and Prommer [24]

Haematological Analyses

The methods of blood collection and analysis used in this study have been described in detail elsewhere.[18] Briefly, full blood counts were conducted using an automated haematology analyser (Sysmex XE-2100, Sysmex Corporation, Kobe, Japan) which was calibrated against appropriate reference materials and checked daily against internal and external quality controls. The full blood count provided direct measures of haemoglobin concentration ([Hb]), reticulocyte percentage (Ret%), red blood cell count (RBC), haematocrit (Hct), mean cell volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and red cell distribution width (RDW). Algorithms described previously by Durussel *et al.* [2] were used to calculated total blood volume (BV), erythrocyte volume (EV) and plasma volume (PV). The OFF-score was calculated as [Hb] – 60 √(Ret%).[10]

Statistical analysis

All values are reported as mean (± SD) unless otherwise stated. Linear mixed models were fitted with all physiological, performance and haematological variables entered as response variables. Group, (measurement) Time, Sex and Sport were included in the models as fixed effects, and subject as a random effect. In addition, where they were found to be appropriate, the models allowed for different within-subject variability for males and females, as well as within-subject autocorrelation.

The raw Ret% values were highly skewed and were square root transformed before all analyses,[25] while Hb_{MASS} was log transformed before all analyses, then back transformed and mean differences expressed as percentages to aid interpretation of the results. All other variables were modelled in their raw form.

Depending upon the (response) variable, all subjects had one or two readings at baseline, at which point no differences between Groups would be expected. As a consequence, the major interest of the analyses was the interaction between Group and Time, rather than their main effects. In particular, components of the interaction were estimated by subtracting the difference between corresponding times for the corresponding PLACEBO group(s) from the difference for the relevant EPO group(s), and are expressed as estimated mean difference [95% confidence interval] unless otherwise stated. All statistical analyses were conducted using the "nlme" package[26] for the statistical program R (version 3.1.0).[27]

In addition to the above analyses, results from each participant's blood samples were entered into a purpose built spreadsheet to assess the sensitivity of the ABP during and after rHuEPO dosing. Previous work has extensively described the methods by which blood parameters are analysed by the ABP.[18] Initially, the ABP algorithms generate tolerance thresholds for [Hb], Ret% and OFF-score based on population data for means and between-subject variances.[28] However, once athlete values are introduced, the algorithm thresholds 'adapt' so each athlete has their own upper and lower limits for acceptable or normal blood values based on the specific within-subject mean and variance. The ABP algorithms were used to determine the number of participants with suspicious (99% specificity, <1 in 100 chance of encountering such a value in a non-doped participant,[14]) and abnormal blood values (99.9% specificity, <1 in 1000 chance of encountering such a value in a non-doped participant,[14]).

RESULTS

Sex and Sport

While main interest was in the interactions between Group and Time, which amount to within-subject comparisons, the fixed effects of Sex and Sport and their interaction, were also considered for each of the 18 response variables. The Sex by Sport interaction was not significant for any of variables with a smallest P-value of 0.119 for plasma volume, while the main effect of Sex was found to be significant ($P < 0.05$) for 11 of the response variables with only three variables (TTE, RPE and Ret%) having p-values > 0.2. For Sport, the only variables with small P-values were VO_{2PEAK} ($P= 0.098$) and TTE ($p < 0.001$); all of the other variables had P-values > 0.2. In the analyses reported below, Sport has only been included for VO_{2PEAK} and TTE.

Placebo

The only "treatment" given to the placebo subjects was iron supplements and sham saline injections. As a consequence it might be expected that there would be little, if any, effect of Group or Time for the two Placebo groups; this was not the case and provides justification for comparing the time differences among the EPO subjects with the corresponding difference among the Placebo subjects. The only variable for which the Group by Time interaction was significant was MCV ($p = 0.005$) while the only variable for which the main effect of Group was significant was RPE ($p = 0.009$). However the main effect of Time was significant ($p < 0.05$) for 12 of the 18 variables with only three variables (VO_{2PEAK}, TTE and Ret%) having p-values in excess of 0.3.

Physiological and performance variables

There was a significant interaction between Group and Time for VO_{2PEAK} ($P = 0.005$, Figure 2). VO_{2PEAK} was significantly elevated relative to baseline for HIGH and COMB groups after the high dose period (0.26 $L \cdot min^{-1}$ [0.12, 0.41], $P < 0.001$) and remained elevated compared with baseline after three weeks rHuEPO micro-dosing for the COMB group (0.25 $L \cdot min^{-1}$ [0.04, 0.45], $P = 0.020$). There was no significant Group by Time interaction for TTE ($P = 0.493$), however there was a trend towards an increase compared with baseline for both groups after the high dose period (28 s [-7, 63], $P = 0.116$) and for the COMB group after the three week rHuEPO micro-dose (28 s [-22, 78], $P = 0.290$). Similarly, the Group by Time interaction was not significant for [La^-] ($P = 0.159$), however there was a significant decrease in [La^-] relative to baseline for both rHuEPO dosed groups after the high dose period (-2.2 $mmol \cdot L^{-1}$ [-4.1, -0.3], $P = 0.026$). The decrease tended to remain for the HIGH group after the four week washout (-2.3 $mmol \cdot L^{-1}$ [-5.0, 0.4], $P = 0.098$). Whilst there was a significant Group by Time interaction for RPE ($P = 0.032$), it remained stable throughout the administration and washout periods and no contrasts reached significance. Similarly, there was no interaction between group and time for peak heart rate ($P = 0.124$) which remained stable throughout the study.

Sensitivity of the ABP

Both regimens of EPO dosing altered the key parameters of the ABP with significant Group by Time interactions for [Hb], Ret% and OFF-score. [Hb] increased relative to baseline for rHuEPO dosed groups after the two week high dose (2.18 $g \cdot dl^{-1}$ [1.54, 2.82], $P < 0.001$) and remained elevated after three and four weeks of washout for the COMB (1.06 $g \cdot dl^{-1}$ [0.05, 2.06], $P = 0.039$) and HIGH groups (1.98 $g \cdot dl^{-1}$ [0.96, 3.00], $P < 0.001$), respectively (Figure 3). Ret% increased compared with baseline in both conditions after the high EPO dose period (0.84 [0.71, 0.97], $P < 0.001$, on the square-root scale) and was still elevated after one week

of micro-dosing for the COMB group (0.31 [0.11, 0.51], P = 0.003, on the square-root scale). However, Ret% then dropped to less than half of the pre-dose percentage through the washout period ($P \leq 0.076$), Figure 3). Conversely, post high dose OFF-score was lower than baseline for rHuEPO dosed groups (-27 [-37, -17], $P < 0.001$), but thereafter was higher than baseline for all subsequent measurements in both groups ($P \leq 0.008$), with the exception of week one of the micro-dose period for the COMB group (Figure 3).

A large number of participants were flagged as having suspicious or abnormal passport values throughout the dosing and washout periods (Table 2). Seventy-five percent of participants in the HIGH and COMB groups were flagged as having a suspicious [Hb] value after the high dosing period, of which 56% were considered abnormal. However, the sensitivity of the [Hb] ABP parameter declined thereafter so that only 19% of participants had suspicious values (13% abnormal) three weeks post the rHuEPO high dose period in both groups (washout week 3 and post micro-dosing respectively for the HIGH and COMB groups). Ret% was slightly more sensitive than both [Hb] and OFF-score after the high dose period when 94% and 88% of rHuEPO dosed participants registered suspicious and abnormal Ret% values respectively. The sensitivity of the Ret% parameter improved during the washout period when all rHuEPO dosed participants registered at least one abnormal value and at least two suspicious values. Comparatively, the sensitivity of the OFF-score ABP parameter was somewhat lower immediately after the high dose period (37.5% suspicious and 13% abnormal) and, in the COMB group, after week one of the micro-dose period. However, when measured two weeks post high dose period in both groups (washout week 2 and micro-dose week 2 for HIGH and COMB groups respectively) 100% of participants were flagged as having suspicious OFF-score values of which 75% were considered abnormal.

Haematological variables

There was a Group by Time interaction for Hct ($P < 0.001$) characterised by a large increase compared with baseline post high-dose for the HIGH and COMB groups (7.9% [6.0, 9.8], $P < 0.001$, absolute change). Although Hct declined thereafter, it remained elevated relative to baseline in HIGH and COMB groups at the conclusion of the washout period ($P \leq 0.044$, Figure 4). Again there was a significant Group by Time interaction for RBC ($P < 0.001$). RBC increased relative to baseline in both HIGH and COMB groups (0.67 $u.L^{-1}$ [0.50, 0.84], $P < 0.001$) post high dosing and remained elevated in both rHuEPO dosed groups four weeks after the final EPO dose ($P \leq 0.005$, Figure 4).

Properties of red cell size and haemoglobin per cell also changed following rHuEPO dosing. There was a significant Group by Time interaction for RDW ($P < 0.001$), MCV ($P < 0.001$) and MCHC ($P < 0.001$). RDW increased relative to baseline following the high dose period

in the HIGH and COMB groups (2.2 % [1.7, 2.7], P < 0.001), and was still elevated after week one of the washout for the HIGH group and after two weeks of micro-dosing for the COMB group (Figure 5). There was a large increase in MCV for the HIGH and COMB groups (3.38 fL [2.18, 4.57], P < 0.001) after the two week high dose which was sustained through to week one of the washout for the HIGH group and after two weeks of micro-dosing for the COMB group. In the HIGH and COMB groups MCHC was lower after high dosing (-8.91 g·L [-13.1, 4.7], P < 0.001), and remained lower in the COMB group after two weeks of micro-dosing. There was also a significant interaction between Group and Time for MCH (P = 0.015), however it remained largely stable and there were no significant differences between baseline and dosing or washout measures for either experimental group (Figure 5).

Blood volumes and Hb_{MASS}

Overall blood volume was stable in both experimental groups throughout the baseline, administration and washout periods and there was no Group by Time interaction (Figure 6). However, there was a significant Group by Time interaction for EV (P < 0.001) and PV (P = 0.002) (Figure 6). The interactions were characterised by a significant increase in EV after the high dose period (540 ml [392, 687], P < 0.001) and a concomitant decrease in PV (-383 ml [-585, -180], P < 0.003) for the HIGH and COMB groups. There was also a significant Group by Time interaction for Hb_{MASS} (P < 0.001). Relative to baseline, there was a large increase in Hb_{MASS} for the HIGH and COMB groups directly after the rHuEPO high dose administration ended (18.4% [12.7, 24.5], P < 0.001) and remained elevated through four and three weeks of the washout period for the HIGH and COMB groups respectively (Figure 4).

DISCUSSION

The results from this study indicate maintenance micro-doses of rHuEPO are sufficient to maintain physiological adaptations and performance improvements following a two week administration of rHuEPO high doses designed to illicit rapid and large increases in [Hb] and Hct. However, the administration of a low dose of rHuEPO did not reduce the detective sensitivity of the ABP with all participants from the COMB dosing group registering ‘suspicious’ or ‘abnormal’ blood values during the micro-dose administration or washout periods.

In line with previous research, VO_{2PEAK} increased following a two week high dose of rHuEPO. The magnitude of the increase was similar to that previously reported following periods of low and moderate dose rHuEPO administration [2-8] and was accompanied by a modest but non-statistically significant improvement in TTE. Unlike previous research,[2,4,29] both VO_{2PEAK} and TTE returned to baseline following a four week washout in the HIGH group. Conversely, administration of rHuEPO micro-doses, equivalent to less than 5% of the

original high dose, was sufficient to maintain physiological and performance improvements observed in the COMB group for three weeks after the final high dose. Russell *et al.* [6] reported a similar preservation of physiological and performance improvements after a five week micro-dose period. While these results are not surprising, they further illustrate the potent effect of rHuEPO and the potential for athletes to benefit from rHuEPO abuse with a somewhat low likelihood of generating an adverse analytical finding using current direct testing techniques, particularly when blood or urine samples are not collected within 15-20 hours of a micro-dose administration.[19]

Physiology and performance changes were accompanied by large changes in a number of haematological variables. High doses of rHuEPO caused a three to four fold increase in Ret%, a 17-19% increase in [Hb] and concomitant increases in Hct, Hb_{MASS} and EV. While these haematological changes provided large beneficial improvements in the oxygen carrying capacity of the blood and in turn oxygen uptake, all rHuEPO dosed participants registered at least one abnormal blood value during the administration or washout periods. Recent studies have reported low sensitivity of the ABP to combination rHuEPO moderate-low dose [30] or micro-dose regimens [18] and suggested athletes may use such techniques to evade anti-doping detection. However, the results of the current study indicate that athletes who use a more aggressive 'boosting' dose will likely register suspicious or abnormal ABP values even if the 'boosting' period is followed by a rHuEPO micro-dose period. In particular, the OFF-score and Ret% were highly sensitive to previous rHuEPO high dosing during the three week micro-dose period and in the initial weeks of a four week washout. Importantly, the results support the use of the ABP as a detection method in isolation and to target direct urine and plasma testing which research suggests may be limited by time of sample collection in their sensitivity to generate adverse analytical findings in athletes using micro-dosing regimens.[19]

Sensitivity of the ABP was also demonstrated by the finding of abnormal and suspicious values for one participant for whom the two week rHuEPO high dose seemed to have minimal beneficial effect on haematology, physiology or performance. The participant, a 25 year old female runner with a VO_{2PEAK} of 2.9 $L \cdot min^{-1}$ (48 $ml \cdot kg^{-1} \cdot min^{-1}$), recorded a post rHuEPO high dose [Hb] increase of 0.4 $g \cdot dl^{-1}$ compared to a group mean increase of 2.18 $g \cdot dl^{-1}$. In clinical populations, initial ESA hyporesponsiveness is defined as a failure to increase [Hb] after one month of weight appropriate ESA dosing [31] and is commonly caused by absolute or functional iron deficiency.[32,33] Administration of rHuEPO in healthy populations can increase the demand for iron such that even in the presence of iron supplementation, available iron is rapidly reduced.[4] Given the participant presented with a relatively low serum ferritin of 16 $umol \cdot L^{-1}$ and transferrin saturation of 32%, it is possible administration of a single IV iron supplement two weeks before rHuEPO high dosing began was insufficient to support accelerated erythropoiesis. However, the small changes in [Hb] and Ret% were still

sufficient to trigger an abnormal Ret% value and a suspicious OFF-score value in the second week of the four week washout period.

Suspicious ABP values were also registered for a participant from the COMB-PLACEBO group during the baseline and washout periods. The participant, a 21 year old female runner, registered a suspicious OFF-score of 26 in the second week of baseline testing derived from a [Hb] of 10.9 $g{\cdot}dL^{-1}$ and Ret% of 1.9%, an increase of 0.4 $g{\cdot}dL^{-1}$ and 0.9% respectively, from the results of the first baseline test. Ashenden *et al.* [18] also described a false positive OFF-score value from a baseline blood test. Whilst the authors couldn't isolate the cause of the false positive value, they suggested the lower than expected Ret% group mean may have been caused by instrument bias despite the instrument running within manufacturer and QC guidelines.[18] Following review of several individual blood profiles by an expert haematologist, instrument bias or error were ruled out as the cause for the suspicious baseline OFF-score value in the present study. Instead, the sudden increase in Ret% (and subsequent drop in OFF-score) was likely caused by the administration of IV iron which occurred between the two baseline measures. Such a scenario is described recently by Garvican *et al.* [34] for runners with compromised iron status. Importantly, while a single isolated suspicious value may be used to identify athletes for further targeted testing, it is not used to initiate anti-doping proceedings [35] and the follow up tests combined with expert review would likely identify the poor iron status as the cause of the suspicious baseline OFF-score.

LIMITATIONS

It is important to note the presence of a number of limitations related to the method employed by the present study. Firstly, blood samples were collected somewhat more frequently than could be expected in a real world situation which may have decreased the sensitivity of the ABP to identify possible rHuEPO dosing.[18] However, given all rHuEPO-dosed participants registered at least one suspicious value during the administration or washout periods, it appears the sensitivity of the ABP was not affected by the weekly collection of blood samples in the present study. Secondly, the pattern and magnitude of rHuEPO dosing and iron supplementation may not reflect actual dosing regimens used by athletes and support staff attempting to avoid detection techniques. Given use of performance enhancing substance abuse is done in a clandestine fashion, accurate replication of real world dosing and supplementation practices is difficult. Thirdly, the micro-dose period was shorter than the wash out period which limits more direct comparison between the responses of the HIGH group during the washout and COMB group during the micro-dose period (i.e. the post high dose response with and without micro-dosing). Finally, the use of recreational athletes as participants, and not well-trained or elite athletes, who may display greater variation in [Hb] and Ret% around periods of heavy training or competition, may have inflated the sensitivity

of ABP parameters. Nevertheless, the results presented in the current study provide important empirical evidence of the physiological and haematological response to regimens of combined high-micro dose rHuEPO administration.

CONCLUSION

This study demonstrated that a rHuEPO micro-dose was sufficient to maintain moderate physiological and performance improvements (VO_{2PEAK} and TTE) induced by a two week administration period of a rHuEPO high dose for up to three weeks. Performance improvement was accompanied by concomitant increases in Hb_{MASS}, [Hb], Hct, RBC and EV that were also maintained for up to three weeks by administration of a rHuEPO micro-dose. However, the majority of participants administered rHuEPO registered [Hb], OFF-score or Ret% values that were flagged as suspicious or abnormal by the ABP. The OFF-score was particularly sensitive in the weeks following a rHuEPO high dose despite one group still being administered a rHuEPO micro-dose. These results indicate there is sufficient sensitivity in the ABP to detect use of high rHuEPO doping regimens in athletic populations and they provide important empirical examples for use by anti-doping experts.

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Table 1. Participant characteristics for each group (mean ± SD). High denotes high doses of rHuEPO; Combined denotes high doses of rHuEPO followed by micro-dosing; and placebo denotes saline injections (See Figure 1).

Group	Number and sex (female/male)	Age (years)	Height (cm)	Mass (kg)	VO_{2PEAK} ($l \cdot min^{-1}$)
High	n = 8 (2F/6M)	28.1 ± 4.6	179.9 ± 13.8	79.8 ± 12.8	4.21 ± 0.91
High Placebo	n = 4 (1F/3M)	34.2 ± 13.4	174.7 ± 5.6	71.7 ± 16.7	3.77 ± 0.93
Combined	n = 8 (0F/8M)	36.3 ± 8.5	179.2 ± 8.5	75.4 ± 5.0	4.24 ± 0.48
Combined Placebo	n = 4 (2F/2M)	33.5 ± 11.4	173.8 ± 4.2	77.5 ± 11.5	3.64 ± 0.49
All	n = 24 (5F/19M)	32.7 ± 9.0	177.8 ± 9.7	76.6 ± 10.9	4.05 ± 0.73

Table 2. Number of participants breaching ABP individual thresholds (data presented as *n*). Sensitivity is either 99% (1:100 false positive) or 99.9% (1:1000 false positive)

	B1	B2	PH	M1	M2	PM	W1	W2	W3	W4
[Hb] 99%										
HIGH (n=8)			5	-	-	-	4	2	1	
HIGH PLC (n=4)				-	-	-				
COMB(n=8)			7	6	4	2				
COMB PLC (n=4)	1									
[Hb] 99.9%										
HIGH (n=8)			4	-	-	-	2	1	1	
HIGH PLC (n=4)				-	-	-				
COMB(n=8)			6	2	3	1				
COMB PLC (n=4)										
Ret% 99%										
HIGH (n=8)	1		7	-	-	-		8	5	3
HIGH PLC (n=4)				-	-	-				
COMB(n=8)	1		8		7	7	7	8	7	2
COMB PLC (n=4)		1								
Ret% 99.9%										
HIGH (n=8)			6	-	-	-		5	5	3
HIGH PLC (n=4)				-	-	-				
COMB(n=8)			8		5	5	7	6	4	1
COMB PLC (n=4)										

OFF$_{score}$ 99%									
HIGH (n=8)		2	-	-	-	2	8	5	3
HIGH PLC (n=4)			-	-	-				
COMB(n=8)		4		8	8	7	5	3	
COMB PLC (n=4)	1						1		
OFF$_{score}$ 99.9%									
HIGH (n=8)		1	-	-	-	2	5	2	3
HIGH PLC (n=4)			-	-	-				
COMB(n=8)		1		7	6	7	5	2	
COMB PLC (n=4)									

B1 and B2 – baseline; PH – post 2-weeks of high dose rHuEPO; M1 and M2 – after 1 and 2 weeks of micro-dose rHuEPO; PM – post 3-weeks of micro-dose rHuEPO; W1, W2, W3 & W4 – after 10, 17, 23 and 30 days after stopping rHuEPO dosing.

Figure captions

Figure 1. Study protocol. A cohort of n = 16 healthy active subjects received rHuEPO treatment, with a further n = 8 control subjects injected with saline instead of rHuEPO. Eight subjects (plus four of the controls) received three weeks of micro-dose treatment (or saline) in addition to the preliminary treatment designed to elevate haemoglobin concentration by 10-15%. All subjects were measured for four weeks after the cessation of their respective dosing regimens (rHuEPO or saline). All subjects received a single intravenous iron treatment (100mg) after the first baseline blood draw.

Figure 2. Physiological and performance variable response to rHuEPO dosing and during a subsequent four week washout. Left panel of each sub-figure displays group mean and SD for HIGH and HIGH-PLACEBO groups as well as individual response for HIGH group. Right panel of each sub-figure displays group mean and SD for COMB and COMB-PLACEBO as well as individual responses for COMB group. ***** denotes significantly greater than baseline ($P \leq 0.05$); † denotes significantly lower than baseline ($P \leq 0.05$).

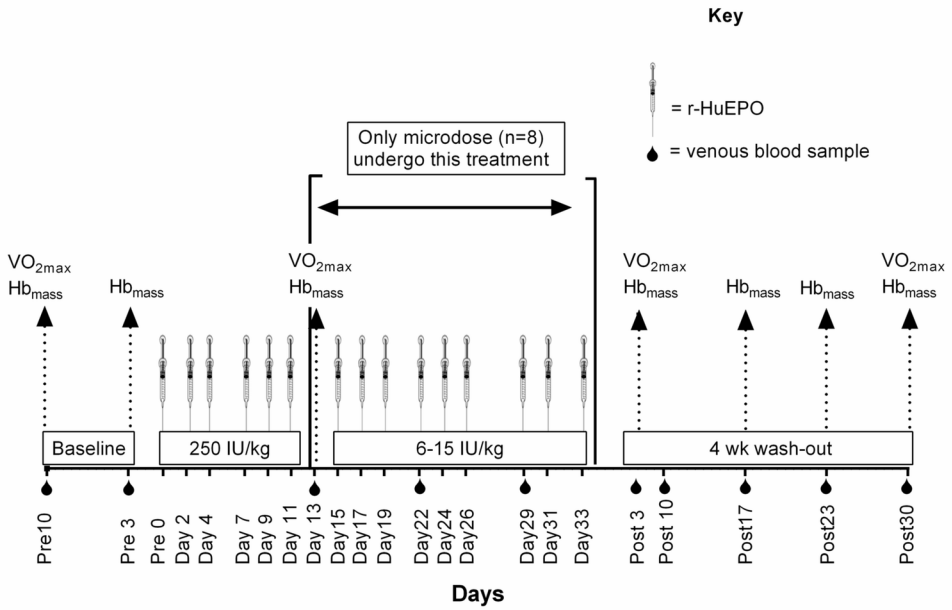
Figure 3. ABP parameter response to rHuEPO dosing and during a subsequent four week washout. Left panel displays group mean and SD for HIGH and HIGH-PLACEBO groups as well as individual response for HIGH group. Right panel displays group mean and SD for COMB and COMB-PLACEBO as well as individual responses for COMB group. ***** denotes significantly greater than baseline ($P \leq 0.05$); † denotes significantly lower than baseline ($P \leq 0.05$).

Figure 4. Hb mass, Hct and RBC response to rHuEPO dosing and during a subsequent four week washout. Left panel displays group mean and SD for HIGH and HIGH-PLACEBO groups as well as individual response for HIGH group. Right panel displays group mean and SD for COMB and COMB-PLACEBO as well as individual responses for COMB group. ***** denotes significantly greater than baseline ($P \leq 0.05$).

Figure 5. Changes red cell size and haemoglobin content in response to rHuEPO dosing and during a subsequent four week washout. Left panel displays group mean and SD for HIGH and HIGH-PLACEBO groups as well as individual response for HIGH group. Right panel displays group mean and SD for COMB and COMB-PLACEBO as well as individual responses for COMB group. * denotes significantly greater than baseline ($P \leq 0.05$); † denotes significantly lower than baseline ($P \leq 0.05$).

Figure 6. Changes in total blood volume, plasma volume and erythrocyte volume in response to rHuEPO administration and during a subsequent washout. Left panel displays group mean and SD for HIGH and HIGH-PLACEBO groups as well as individual response for HIGH group. Right panel displays group mean and SD for COMB and COMB-PLACEBO as well

as individual responses for COMB group. ***** denotes significantly greater than baseline ($P \leq 0.05$); † denotes significantly lower than baseline ($P \leq 0.05$).



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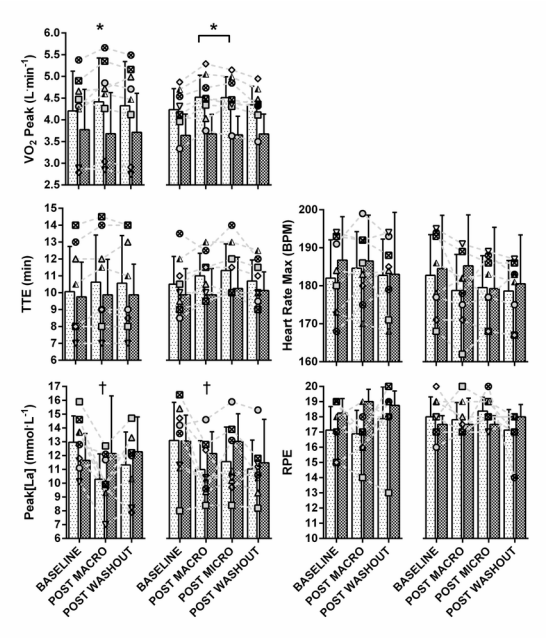

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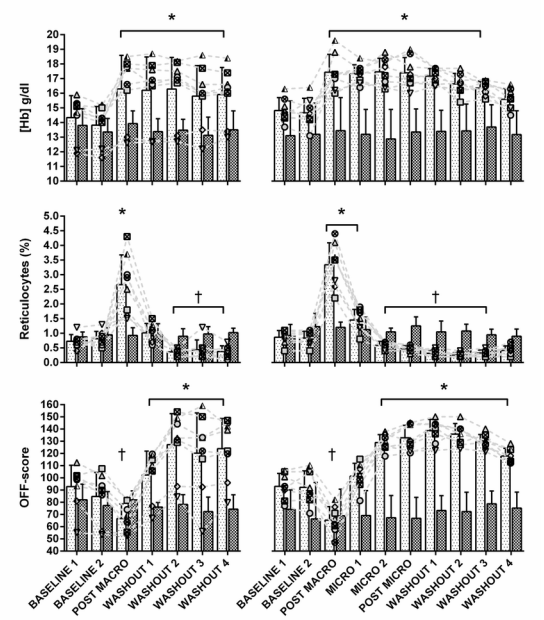

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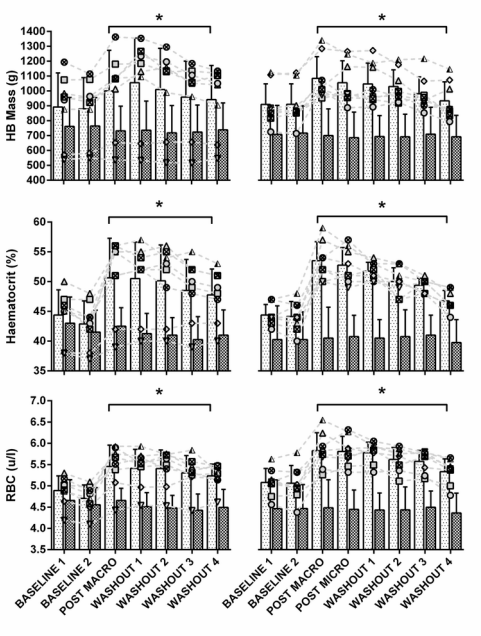

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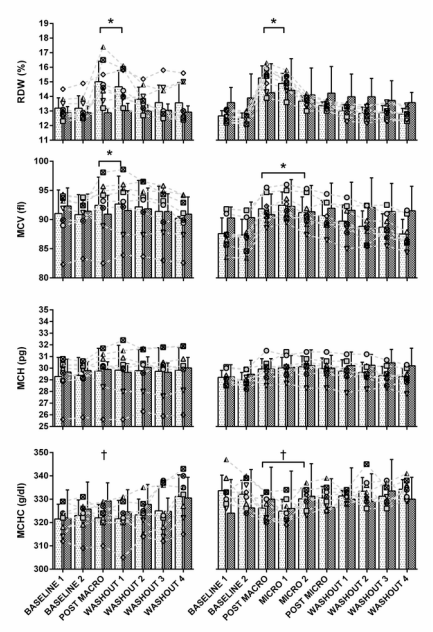

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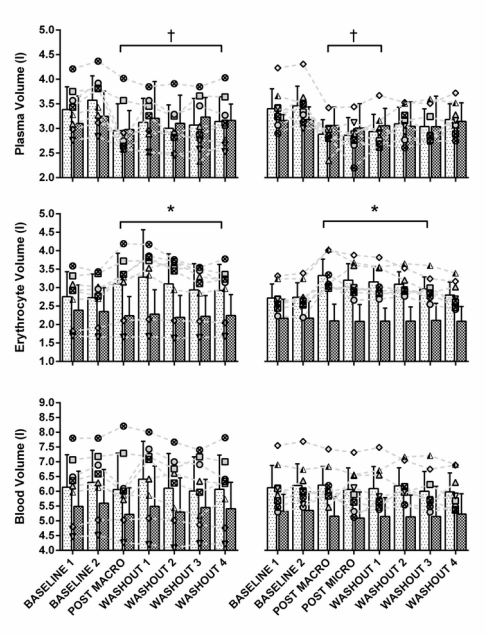

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