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Running Title: THE ERGOGENIC EFFECTS OF MICRO-DOSES OF EPO

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ABSTRACT

Purpose: We investigated the effects of recombinant human erythropoietin (rHuEPO) administration on exercise endurance, maximal aerobic performance and total hemoglobin mass (tHb). We hypothesized that frequent, small intravenous injections of epoetin β would increase time trial performance, peak oxygen uptake ($\dot{V}O_{2peak}$) and tHb in both males and females.

Methods: We included 48 healthy, recreational to trained males (n=24, mean ± standard deviation $\dot{V}O_{2peak}$ of 55±5 ml O$_2$×kg$^{-1}$×min$^{-1}$) and females (n=24; $\dot{V}O_{2peak}$ of 46±4 ml O$_2$×kg$^{-1}$×min$^{-1}$) in a counter-balanced, double-blind, randomized, placebo-controlled study design stratified by sex. Time trial performance, $\dot{V}O_{2peak}$, and tHb were determined before and after intravenous injections of either rHuEPO (9 IU×kg bw$^{-1}$ epoetin β) or saline (0.9% NaCl) three times weekly for 4 weeks.

Results: A time × treatment effect (P<0.05) existed for time trial performance. Within the rHuEPO group, mean power output (MPO) increased by 4.1 ± 4.2% (P<0.001). Likewise, a time × treatment effect (P<0.001) existed for $\dot{V}O_{2peak}$, where the rHuEPO group improved $\dot{V}O_{2peak}$ and peak aerobic power by 4.2 ± 6.1% (P<0.001) and 2.9 ± 4.0% (P<0.01), respectively. A time × treatment effect (P<0.001) existed for tHb, where the rHuEPO group increased tHb by 6.7 ± 3.4% (P<0.001). A main effect of ‘sex’ alone was also evident (P<0.001) but no sex-specific interactions were found. No changes were observed in the placebo group for MPO, $\dot{V}O_{2peak}$, peak aerobic power or tHb.

Conclusion: Micro-doses with intravenous rHuEPO provide a sufficient erythropoietic stimuli to augment tHb and enhance aerobic-dominated performance in both trained males and females.

Key Words: DOPING, ELITE-SPORT, HEMATOLOGY, ENDURANCE, BLOOD DOPING
INTRODUCTION

In recent years, the ergogenic effects of recombinant human erythropoietin (rHuEPO) on endurance performance has been questioned and disputed (1, 2) despite a strong physiological rationale for rHuEPO misuse to be performance enhancing (3) and decades of athlete testimonies and doping scandals involving EPO (4, 5). Currently, only three studies have investigated the effect of rHuEPO on endurance performance using the most competition relevant test regime, i.e., a closed-end time-trial protocol (6-8). Two of these studies reported a 5-6% faster 3,000 m running performance following four weeks of subcutaneous 50 IU rHuEPO×kg body weight (bw)⁻¹ every second day in highly trained runners (6, 7). However, both studies suffer from their non-placebo controlled and open-labeled study design. The third study utilized a randomized, double-blinded placebo-controlled design and reported no effect of eight weeks of 6,000 IU subcutaneous rHuEPO injections per week (~80 IU×kg bw⁻¹) on road race or laboratory time trial performance in well-trained male cyclists (8). Road race performance was assessed during an uphill time trial (~100 min) preceded by 110 km cycling (8). Despite a commendable attempt to evaluate human endurance performance with high ecological validity in a strong study design, the study received comprehensive critique (9-11), as the road race was only completed after treatment, why no changes of individual performance could be inferred. Furthermore, in elite sports, the typical margin of victory is minimal (~0.3-1%) (12), why an emphasis on reliability is paramount and often secured through highly controlled and familiarized experiments (13). An inadequate familiarization with the testing regime may also explain why Heuberger et al. (8) surprisingly found no differences in the laboratory time trial, as the time trial was performed below the calculated lactate threshold, which could indicate inadequate experience with the testing regime. Thus, despite decades of rHuEPO misuse in elite
sport the effect on performance is still controversial. Accordingly, a randomized controlled trial investigating the effects of rHuEPO on a closed-end time trail with high sensitivity and standardized, relevant and familiarized exercise tests is needed.

A point of extreme importance is the pronounced bias toward male athletes in the existing studies(3). Sex is a basic variable but as of today, only one study examining the ergogenic effects of rHuEPO included an approximately equal distribution of males and females (n=12 vs. n=9, respectively)(14). However, no sex-specific analyses were performed in that study probably due to the relatively small population. In addition, the two remaining studies including female participants enrolled two and five females and with a total of nine and 24 participants, respectively, also precluded sex-specific analyses(15, 16). The need for sex-specific considerations in research is evident and also highlighted by several entities, e.g. the National Institute of Health(17). From a physiological perspective, sex-specific analyses in the context of rHuEPO misuse appear relevant, since testosterone stimulates erythropoiesis(18) and estrogens possibly inhibit erythropoiesis(19), which may favor the response in males. Also, a meta-analysis estimated the blood doping prevalence to be higher among female than male participants (~22% and ~15%, respectively), at the 2011 World Athletics World Championships(20).

Finally, micro-dosing of rHuEPO is a likely current challenge for anti-doping authorities(21). Frequent intravenous injections of low-dose rHuEPO minimize the detection window as the plasma half-life varies between 4-9h after intravenous administration in comparison with >24h after subcutaneous injections(22). For example, a former top-class cyclist admitted to injecting 400-500 IU (~6-8×kg bw\(^{-1}\)) rHuEPO every night(23). Indeed, we recently reported as little as 11 injections of 20 IU rHuEPO×kg bw\(^{-1}\) increase hemoglobin concentration(24) and hematocrit(25).
Thus, with the aim to improve anti-doping efforts, it is relevant to determine the potential performance-enhancing effects of rHuEPO micro-dosing in a sex specific context. Therefore, this study aimed to investigate the effect of a relevant and likely real-world doping scenario on performance in males and females. We hypothesized that as little as 9 IU×kg bw\(^{-1}\) epoetin β given intravenously three times per week for four weeks increase time trial performance, peak oxygen uptake (\(\dot{V}O_2\text{peak}\)) and total hemoglobin mass (tHb) in both males and females.

**METHODS**

**Participants**

We included 48 healthy, recreational to well-trained males (n=24) and females (n=24) (Table 1). Participants were informed both orally and in writing of potential risks and discomforts associated with participation before a written consent was obtained from each participant. The local ethics committee of Copenhagen, Denmark (H-18013069) approved the study performed, which was conducted in accordance with the Declaration of Helsinki and registered on www.clinicaltrials.gov (NCT04965961). The study was conducted at the Department of Nutrition, Exercise and Sports, University of Copenhagen, Denmark, from August 2019 to May 2021.

**Study design**

We utilized a counter-balanced, double-blind, randomized, placebo-controlled study design (Fig. 1), with a randomization algorithm (www.randomizer.org) assigning participants (1:1) to a rHuEPO or placebo group stratified by sex (Table 1). The study consisted
of a two-week baseline period followed by a four-week treatment period. Time trial performance and cardiopulmonary exercise test (CPET) on a cycle ergometer as well as tHb was assessed before and after treatment. Participants were instructed to maintain their usual training regime throughout the study and body composition was determined before and after treatment using dual x-ray absorptiometry (Lunar iDXA, GE Healthcare). Importantly, participants were instructed that any participation in organized sports three months following the last injection was prohibited. The primary outcome was time trial mean power output (MPO) and time to completion. Secondary outcomes were tHb, intravascular blood volumes and hematocrit as well as heart rate, blood metabolite levels, $\dot{V}O_2$, $\dot{V}CO_2$ and the respiratory exchange ratio (RER) during the preload and time trial. For the CPET, the primary outcome was $\dot{V}O_2$peak and the secondary outcome was incremental peak power output (IPPO).

**Assessment of eligibility criteria**

Blood pressure was measured after >5 min of rest in a seated position with the back supported and the feet flat on the floor. Hereafter, all participants completed an initial CPET as detailed below to determine $\dot{V}O_2$peak. Inclusion criteria were a relative $\dot{V}O_2$peak of at least 45 or 50 ml O$_2$×kg$^{-1}$×min$^{-1}$ for male and female participants, respectively. Exclusion criteria were hypertension, diseases requiring prescribed medication and professional athletes.

**Pharmacological intervention**

Following the baseline period, participants received three weekly intravenous injections of 9 IU×kg bw$^{-1}$ epoetin β (NeoRecormon, Roche, Basel, Switzerland) or saline (0.9% NaCl) for four weeks, while blindfolded. Injections were administered by scientific personal not
involved in recruitment or exercise testing. Blood pressure (Omron Healthcare, Kyoto, Japan) and hematocrit (Sysmex XN-450, Kobe, Japan) were measured 48 hours after 2, 5, 8, and 11 injections as well as three, five, and 10 days after last injection. During the treatment, all participants received open-label daily oral doses of 80 mg iron (ferric sulphate) (Tardyferon, Pierre Fabre Pharma GmbH, Germany) and received standard instructions about concomitant food intake. Adherence to iron supplementation was controlled by weekly asking the subject whether they had ingested the tablets.

**Pre and post intervention assessment overview**

Participants were familiarized twice with the time trial protocol during baseline, before conducting a CPET and time trial test on two separate days interspersed by ≥24 hours in the week prior to the first injection. The CPET conducted at the screening day functioned as a familiarization test for the CPET conducted before and after the injection period. Three and five days following the last injection time trial and CPET performance was assessed again, respectively. Determination of tHb was conducted in the last baseline week prior to the treatment period as well as three days after last injection.

**Experimental procedures**

Blinded personnel performed all exercise tests, which began with a standardized warm-up (males: 5 min @ 90W and 150W, females: 5 min @ 75W and 125W) on an electronically braked bike (Monark 839E, Varberg, Sweden or Lode Excalibur Sport, Groningen, Netherlands) using commercially available software (Monark Testing Software version 1.0,15.0 or Lode Ergometry Manager 10). All equipment were calibrated prior to each test. Heart rate was
determined using wireless heart rate straps (Polar, Kempele, Finland). A period of 3-5 min separated the warm-up and the test. The participants were instructed to refrain from caffeine and alcohol intake on the day of testing and to prepare similarly for all test days.

**Time trial performance.** Endurance performance was determined by a 400-kcal time trial preceded by a 60 min submaximal exercise period (denoted preload) at a rating of perceived exertion of ~13 (“somewhat intense”) on the Borg scale corresponding to ~55% of IPPO. Subjects were instructed to prepare identically before all tests and to refrain from exercise the day before each exercise test. Carbohydrate drinks (431 ± 60 ml) were provided during the preload as previously described(26). Water *ad libitum* was available during both the preload and time trial. During the preload, pulmonary variables were measured from the 45-55<sup>th</sup> minute using the same equipment as during the cardiopulmonary exercise test. Thus, carbohydrate and fat oxidation during exercise were assessed via indirect calorimetry. Participants were instructed to complete the time trial as fast as possible. The time trial was initiated by applying a random load of 20 to 25 Newtons (N) by the blinded personnel. The subjects freely selected pedaling cadence, and were able to continuously adjust the workload through verbal communication or by indicating with hand gestures to increase or decrease braking resistance by steps of 1 N. Once the subject reached 400 kcal, the test was terminated. The subjects were blinded to elapsed time and heart rate but received continuous verbal feedback on completed work. Furthermore, the subjects were verbally encouraged during the trial. Blood capillary samples were obtained from a fingertip using 95 μl preheparinized tubes (Clinitubes; Radiometer, Brønshøj, Denmark) before, halfway and immediately after completion during both the preload and the time trial. Samples were analyzed using an ABL 800 blood gas analyzer (Radiometer, Brønshøj, Denmark) for blood lactate and glucose concentration, pH, plasma sodium, potassium, and bicarbonate concentration.
Cardiopulmonary exercise test. IPPO and \( \dot{V}O_2 \text{peak} \) were determined using an exhaustive incremental cycling starting at 150 W for males and 125 W for females followed by increments of 25 W × min\(^{-1}\). The highest 30 s average \( \dot{V}O_2 \) was defined as \( \dot{V}O_2 \text{peak} \) and determined from expired breath-by-breath O\(_2\) and CO\(_2\) fractions and ventilation (Quark b2, Cosmed, Rome, Italy). Cadence had to be maintained between 70 rpm and 100 rpm. Exhaustion was reached when cadence could not be maintained above 70 rpm or when a participant terminated the test. IPPO was calculated as \( W_{\text{compl}} + 25 \times (t/60) \) (\( W_{\text{compl}} \), last completed workload [W]; \( t \), time at last workload before exhaustion [s]).

Total hemoglobin mass and intravascular volumes. Red blood cell volume (RBCV), plasma volume (PV), total blood volume (BV), and tHb were measured in duplicates separated by 0-24 h using the carbon monoxide (CO)-rebreathing technique(27). At least 24 h separated the measurement and the next performance evaluation. Briefly, participants were placed in a supine position with legs raised, after which four capillary blood samples from a fingertip were collected in 35μl preheparinized tubes (safeClinitubes, Radiometer, Brønshøj, Denmark) and analyzed for percent carboxyhemoglobin on an ABL 800 blood gas analyzer (Radiometer, Brønshøj, Denmark). The participants then inhaled 1.0 ml × kg bw\(^{-1}\) chemically pure (99.997%) CO (CO N47; Air Liquide, Paris, France) delivered via a 100-ml plastic syringe (Omnifix; Braun, Melsungen, Germany) to a custom designed spirometer (Hans Rudolph, Kansas, USA) creating a closed system. The system encompassed 5 L of 100% oxygen, which was rebreathed for 2 min. Four capillary blood samples were collected and analyzed nine minutes after the inhalation of CO using the same technique used during baseline sampling. A CO analyzer (Draeger, Lübeck, Germany) was used to evaluate whether a leak in the closed system occurred.
during the rebreathing period and to measure any leftover CO in the spirometer and end-tidal CO before and 3 minutes after the rebreathing period. The hematocrit, hemoglobin concentration and the difference in percent carboxyhemoglobin were used to calculate tHb and intravascular volumes(27), which was adjusted for a loss of CO to myoglobin of 0.3% per minute(28) and through ventilation by multiplying the difference in end-tidal CO from before to after rebreathing with an estimated alveolar ventilation of 5 liter per minute(27).

**Statistics and power calculation**

Four weeks of 50 IU×kg bw⁻¹ every second day improve (mean ± standard deviation (SD)) 3.000 m running performance and \( \dot{V}O_2 \) peak by 4.6 ± 2.4% and 5.8 ± 8.0%, respectively(6). We assumed a smaller effect following 9 IU×kg bw⁻¹ and based our power calculation for detecting a difference of 3% in both outcomes. Accordingly, using a two-tailed t-test, a power of 0.80 and a significance level of 5%, a sample size of 23 was required to be adequately powered for detection of 3% changes, assuming a SD of 4%. When taking into account a 5% attrition rate, 24 participants were enrolled in both groups. Considering the MPO in a comparable population during a similar exercise protocol(26), it is expected to enable a detection of changes in MPO by ~9W. For tHb, inclusion of 48 participants allow a detection of differences of more than 2% based on previous findings(29).

SPSS was used for statistical analyses (IBM SPSS Statistics, version 26.0.0) with the level of significance set at P<0.05 and P<0.10 was considered as a statistical tendency. The data are presented as means ± SD. Anthropometric characteristics were evaluated using an unpaired t-test. A linear mixed model for repeated measures was used to assess the effect on
performance measures with fixed effects of time (pre vs. post), treatment (rHuEPO vs. placebo), sex (male vs. female), and all their interactions. Participant number was used to identify repeated measures and to define a random factor. Significant main effects for any interaction were followed by a Sidak-adjusted pairwise comparison. Linear regression analysis was performed to assess the associations between hematocrit, tHb and exercise performance. The coefficient of determination ($r^2$) was interpreted using a scale of magnitudes (30), where $r^2 < 0.01$ is interpreted as trivial, 0.01–0.09 as small, 0.09–0.25 as moderate, 0.25–0.49 as strong, 0.49–0.81 as very strong and >0.81 as nearly perfect.

The coefficient of variation (CV%) of the performance tests was calculated by dividing the SD of the differences between the pretreatment and posttreatment ($\overline{X}$) in the placebo group by the grand mean ($\overline{X}$) and dividing the quotient by $\sqrt{2}$: $CV = (\overline{X} / \overline{X}) / \sqrt{2}$. Regarding the CO-rebreathing measurements, a CV% was calculated both before and after treatment using the difference between the two duplicate measurements pretreatment and posttreatment, respectively. Finally, blinding efficiency was evaluated by letting participants guess their treatment and the Bang blinding index(31). The index ranges from -1 or 1 and if the 95% confidence interval (CI) included null, the blinding was maintained.

RESULTS

Baseline characteristics were not different between treatment groups (Table 1). Likewise, body composition before and after the treatment period was not statistically different between groups, except for the female placebo group, who had a 6% lower body weight than the female rHuEPO group (main effect of group, P<0.05; see Supplemental Table 1, Supplemental
Digital Content, Body composition before and after recombinant human erythropoietin, http://links.lww.com/MSS/C744). No adverse events during or following rHuEPO treatment were observed and blood pressure was not different between groups at all times (see Supplemental Table 2, Supplemental Digital Content, Mean ± SD values for systolic and diastolic blood pressure during and 3, 5, and 10 days after recombinant human erythropoietin or placebo treatment, http://links.lww.com/MSS/C744). The Bang blinding index was 0.08 (95% CI, -0.74 to 0.40) for the rHuEPO group and 0.08 (95% CI, -0.66 to 0.49) for the placebo group, demonstrating a successful blinding. The CV% and the corresponding minimal detectable difference in parentheses were 2.7% (6.6W) and 2.0% (29s) for time to completion and MPO during the time trial, while CV% for \( \dot{V}O_{2\text{peak}} \) and \( tHb \) were 2.4% (85 ml O\(_2\)) and 0.9% (7g), respectively. Adherence to oral iron supplements was 95%. Gastrointestinal side effects were reported by 18 participants (12 females, 6 males), who were advised to ingest the tablets only every other day, and for one male and one female subject, this was modified to every third day.

**Endurance performance**

**Preload.** Preload MPO, pulmonary variables including respiratory exchange ratio (RER), and heart rate were not different between the rHuEPO and placebo group before and after treatment, respectively (see Supplemental Table 3, Supplemental Digital Content, Mean ± SD values for pulmonary data, heart rate and mean power output before and after treatment with recombinant human erythropoietin or placebo, http://links.lww.com/MSS/C744). Likewise, no time × treatment interaction existed for blood metabolites or ions (see Supplemental Table 4, Supplemental Digital Content, Mean ± SD values for blood metabolites and ions before and after treatment with recombinant human erythropoietin or placebo, http://links.lww.com/MSS/C744) during the preload and time trial.
**Time trial performance.** Analysis of MPO and time to completion during the time trial performance revealed a significant time × treatment interaction for both variables (P<0.05) with no difference between groups at baseline (Fig. 2). MPO and time to completion were increased by 4.1 ± 4.2% (10 ± 9W; P<0.001) and 4.3 ± 4.2% (64 ± 60s, P<0.001) in the rHuEPO group, respectively, whereas no changes were observed in the placebo group. A main effect of ‘sex’ was evident (P<0.001) with males producing 98 ± 44W more than female participants.

**Peak aerobic capacity**

A time × treatment effect (P<0.001) and main effect of ‘sex’ (P<0.001) existed for $\dot{V}O_{2peak}$ as well as for IPPO (P<0.05). $\dot{V}O_{2peak}$ and IPPO were improved by 4.2 ± 6.1% (194 ± 220 ml; P<0.001) and 2.9 ± 4.0% (13 ± 14W; P<0.01) in the rHuEPO group, respectively (Fig. 3). There were no differences in $\dot{V}O_{2peak}$ or IPPO between groups at baseline. No differences were evident in the placebo group.

**Total hemoglobin mass and intravascular volumes**

A time × treatment effect (P<0.001) existed for tHb, where the rHuEPO group increased their tHb by 6.7 ± 3.4% (54 ± 24g; P<0.001; Fig. 4). RBCV and BV increased by 7.6 ± 5.1% (176 ± 106 ml; P<0.001) and 2.0 ± 6.1% (110 ± 302 ml; P<0.05), respectively, while PV was unaltered (see Supplemental Fig. 1, Supplemental Digital Content, Mean values for red blood cell volume, plasma volume and total blood volume in histograms with individual participants as lines in females, males, and pooled data from all before and after treatment with recombinant human erythropoietin or placebo, http://links.lww.com/MSS/C744). A main effect of ‘sex’ alone was evident (P<0.001) but no sex-specific interactions were found.
**Hematocrit**

A time × treatment effect and time × treatment × sex effect (P<0.001) existed for hematocrit, which peak increase of 2.6 ± 1.9 percentage-points above baseline values was evident three days after the last injection (Fig. 5). This increase was also present ten days after last injection in the female participants (P<0.05), while no difference was evident in males. On average, males had 3.3 ± 0.7 percentage-points higher (P<0.001) hematocrit than females.

**Association between tHb, hematocrit and performance**

A moderate, positive correlation existed between the change in tHb and MPO (r²=0.19, P<0.05), whereas a statistical tendency for a moderate, positive correlation were found between the change in tHb and time to completion (r²=0.12, P<0.10). No significant correlations was found between the change in tHb and $\dot{V}O_2$peak (r²=0.01; Fig. 6). In addition, no correlations were found for the change (pre-post) in hematocrit for either MPO (r²=0.06), time to completion (r²=0.07) or $\dot{V}O_2$peak (r²=0.00).

**DISCUSSION**

This study demonstrates that four weeks of only 9 IU×kg bw⁻¹ epoetin β injected intravenously can enhance aerobic-dominated performance of trained individuals, thus confirming athlete testimonies of rHuEPO as a potent performance-enhancing agent even at micro-doses. The apparent effect of rHuEPO was irrespective of sex. Our finding that frequent injections of epoetin β enhances performance underlines the necessity for continuous refinement of anti-doping strategies and confirms the solid physiological rationale for even small increases in erythropoiesis results in performance enhancing effects.
Low-dose, frequent injections of rHuEPO enhances endurance and peak performance.

The majority of studies investigating endurance performance following rHuEPO applied open-end testing regimes such as time to exhaustion cycling (32-35), which are less relevant for real-word competitions as they are most frequently distance limited. However, a few closed-end time trial investigations, e.g. time to complete a fixed distance or kilojoules, exist regarding rHuEPO and they are all uncontrolled (6, 7) or highly critiqued (8). Accordingly, we determined closed-end endurance performance in a well-controlled and well-powered design. The enhanced preloaded time trial performance by 4-5% with rHuEPO, corresponding to 10W or 0.21W×kg⁻¹, is in alignment with Haile et al who observed a 5-6% improvement of 3,000m running performance with epoetin β self-administered subcutaneously at 5-fold the doses used in the present study (6). Thus, counterintuitively there seems to be no clear relationship between the administered rHuEPO dose and the performance enhancing effect hereof across studies. This may be related to the different exercise modalities (cycling vs. running), the length of the time trial (25 vs. 10 min), the inclusion of a preload vs. no preload, cardiorespiratory fitness (50 vs. 60 ml O₂×min⁻¹×kg⁻¹), or sex (males vs. females). Furthermore, it should be emphasized as evident from Fig. 2 that three subjects in the rHuEPO group failed to improve their TT performance.

When investigating endurance performance in relation to competitive sports and athletes, the best option is to mimic a real-life race. However, as such setup ultimately has too little control of e.g., weather conditions as well as drafting and pacing strategies, which may severely impact performance and reliability, a controlled laboratory time trial is the most appropriate approach although it may have lower ecological validity compared to a real race situation as previously attempted (8). Herein we applied a 1 h preload preceding the time trial to
better mimic real-life race conditions. The preload is expected to induce some fatigue while reducing glycogen stores by ~50%(36), similar to what is observed in endurance based sports. The time trial test in the present study had low variability and the observed performance-enhancing effects were significantly higher than the corresponding minimal detectable difference. This is likely due to the familiarization of all participants ensuring that they were accustomed with the testing regime, which previously has been argued to be crucial when assessing sport performance(12, 37). Indeed, the CV% for time trial MPO was 3.8% between the two familiarization trials and decreased to 2.7% between the pre and post-test in the placebo group.

The applied micro-dosing regimen increased tHb and RBCV by as much as ~6.5% and ~7.6%, respectively. This was more than expected based on previous studies. Notably, a uniform increase in tHb across the included participants regardless of sex was observed. 20 of 24 the rHuEPO-treated participants experienced >4% increase in tHb. Previously, an increase of similar magnitude in RBCV was evident after four weeks of 35 IU×kg bw⁻¹ epoetin α injected 2-3 times weekly(33), while injecting 50 IU×kg bw⁻¹ epoetin α subcutaneously three times weekly for 25 days in iron-replete males has been reported to increase tHb ~7%(38). One explanation may be the pharmacological difference between epoetin α and β as the half-life after intravenous administration of epoetin β was 20% longer than epoetin α(39), thus providing a sustained erythropoietic signal. However, the rHuEPO dosage applied is not necessarily the sole key determinant of the tHb response, as a comparable 50 IU×kg bw⁻¹ protocol increased tHb by 12% using epoetin α(38) and even 20% when oral iron supplementation was provided and epoetin β was administered(7). Similarly, the 4-5% increase in VO₂peak observed in the present study also
appear pronounced compared with previous studies when considering the dose applied here. An increase in tHb of 1g has been estimated to result in a concomitant increase in \( \dot{V}O_{2\text{peak}} \) of 4 ml\(^4\), why the present increase in tHb of ~54g is expected to increase \( \dot{V}O_{2\text{peak}} \) by ~216 ml. This aligns with the observed group mean increase of 194 ml suggesting that the observed alteration in \( \dot{V}O_{2\text{peak}} \) is a valid, physiological effect induced by the administration protocol (please see a discussion of the individual association in section 4.3). Previous studies applying a 5-fold higher dose of epoetin \( \alpha \) or \( \beta \) injected 2-3 times per week for 25-28 days have reported an increased \( \dot{V}O_{2\text{peak}} \) of ~6-7\%(6, 38, 41). As there were no differences in body composition before and after the injection period (see Supplemental Table 1, Supplemental Digital Content, Body composition before and after recombinant human erythropoietin, http://links.lww.com/MSS/C744), the observed increase in absolute \( \dot{V}O_{2\text{peak}} \) naturally resulted in a parallel relative \( \dot{V}O_{2\text{peak}} \) increase of 2.7 ± 3.0 ml\( \times \)kg\(^{-1} \)\( \times \)min\(^{-1} \) (P<0.01).

We found no differences in whole body substrate utilization despite a numerical difference in RER of 0.03 (Table 3), which could indicate an increased carbohydrate utilization during the preload following rHuEPO treatment. Applying a simple paired \( t \)-test before and after treatment in the rHuEPO group revealed a significant increase in carbohydrate oxidation (P<0.01) while no difference was apparent in the placebo group. This is in contrast with a previous study, where a 56\% increase in fat oxidation was found following a four-week rHuEPO treatment with three weekly subcutaneous injections of 50 IU kg\( \times \)bw\(^{-1} \) in six moderately trained males(41). However, no differences in substrate utilization or systemic fat oxidation following rHuEPO administration could be detected in other trials(42). Thus, our results do not support the notion that rHuEPO treatment in trained males and females alter systemic substrate utilization during submaximal exercise.
No sex-specific differences

In the present study, the observed effects of rHuEPO on performance were independent of sex. Likewise, we found no sex-specific differences in tHb emphasized by the uniform increases. Previously, no studies have been adequately powered to assess whether physiological and ergogenic sex-specific differences exist following rHuEPO treatment. Here, it was expected that the absolute levels of performance and exercise intensity were superior in males compared with females, exemplified by the ~30% faster performance in time trial performance in males compared to females following rHuEPO treatment. In one female participant receiving rHuEPO, we did not observe an increase in tHb (+5 g following treatment). A major physiological difference between males and females with a potential influence on tHb is iron homeostasis. Indeed, ferritin levels are in general higher in males compared to females(43) and the prevalence of iron deficiency and anemia are higher among females, where 32% of exercising females have a history of anemia and 50% have supplemented with iron(44). In addition, 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(32). Notwithstanding, taking the small rHuEPO dosage into account as well as the uniform increases in tHb in the remaining participants as well as an augmented hematocrit of 1.8 percentage point in that specific female participant, we argue that iron deficiency as a decisive variable in the present study, is of little concern.

Does increased tHb explain the increased endurance performance?

As tHb is a predictor of aerobic performance(45), we investigated whether any associations between the changes in tHb and hematocrit and performance were evident (see
Supplemental Fig. 1, Supplemental Digital Content, Mean values for red blood cell volume, plasma volume and total blood volume in histograms with individual participants as lines in females, males, and pooled data from all before and after treatment with recombinant human erythropoietin or placebo, http://links.lww.com/MSS/C744). We only found a positive correlation between the change in tHb and MPO ($r^2=0.19$). It has been suggested that rHuEPO exerts its main effects on $\dot{V}O_2\text{peak}$ through a tHb-induced increase in hemoglobin concentration and thus a higher $C_aO_2$ and peak $O_2$ delivery(46). Our results demonstrate that $\sim19\%$ of the change in endurance performance may be ascribed to the change in tHb, whereas less can be directly described to an increased hematocrit, although it is clear that the present rHuEPO-induced increase in hematocrit may be insufficient to provide a robust signal-to-noise ratio, why caution should be taken when interpreting the correlation. A plethora of variables determine performance and although our CV% for both tHb and time-trial measurements was low (0.9% and 2.7%, respectively), we cannot exclude that measurement errors and biological variation influenced the results as we investigate small effects. Notwithstanding, our findings coincide with reports of $\sim10\%$ of the difference in a time to exhaustion test could be explained by an increased RBCV following rHuEPO treatment(33). In addition, although we observe a small increase in BV of $\sim75\text{ ml}$, cardiac output is not likely to have changed with rHuEPO treatment as both submaximal and maximal cardiac output was unchanged following a 5-6 times higher dose epoetin $\beta$ treatment(46). Furthermore, as rHuEPO administration neither induce angiogenesis(47) or improve oxidative capacity of the skeletal muscle(48), although unclear(49), an augmented arterial $O_2$ concentration likely results in a higher $O_2$ gradient from capillaries to exercising muscles thus allowing an augmented $O_2$ diffusion rate(50). It may also be hypothesized that an increased blood flow to exercising muscles was present, as the time trial MPO in the present
study was performed at ~71% of IPPO, and leg blood flow have been reported to increase when cycling at an intensity of ~80% of IPPO after rHuEPO(51). Thus, for the rHuEPO-induced increase in arterial O$_2$ concentration to increase performance without affecting the abovementioned variables, the arterial-venous O$_2$ difference and/or blood flow in the active muscle groups must be increased. Other explanations may relate to the increase in erythrocytes per se, where we observed a ~7.5% increase in RBCV following treatment (see Supplemental Fig. 1, Supplemental Digital Content, Mean values for red blood cell volume, plasma volume and total blood volume in histograms with individual participants as lines in females, males, and pooled data from all before and after treatment with recombinant human erythropoietin or placebo, http://links.lww.com/MSS/C744). Erythrocytes also function as extracellular buffers via the expression of membrane-bound transport systems such as the AE1 isoform of the anion-exchange transporter and the MCT1 isoform of the monocarboxylate transporter (La/H$^{+}$ co-transporter), which has been found to increase after rHuEPO treatment(52), as well as the cytoplasmic enzyme carbonic anhydrase, which catalyzes the reversible hydration of carbon dioxide to bicarbonate(53). Thus, an increased RBCV could hypothetically increase the buffer capacity by a factor of ~1:1 thereby allowing for a greater degree of anaerobic contribution during a submaximal primarily aerobic-dominated performance test. However, we found no differences in lactate, pH or HCO$_3^{-}$ during the preload (see Supplemental Table 4, Supplemental Digital Content, Mean ± SD values for blood metabolites and ions before and after treatment with recombinant human erythropoietin or placebo, http://links.lww.com/MSS/C744), and since a 9-10% increase in tHb does not improve repeated anaerobic sprint performance(29, 54), it appears unlikely that the observed increase in RBCV improves the buffer capacity to a relevant extent.
Detection possibilities

The present manuscript evaluated the physiological and performance-enhancing effects following a micro-dosing rHuEPO treatment. However, it is important to emphasize that the applied rHuEPO protocol provides a high probability of being detected by current doping tests. Following six subcutaneous injections of 9 IU×kg bw⁻¹, direct rHuEPO tests are capable of detecting rHuEPO in blood and urine samples up to 72 h (91% sensitivity) after last injection(55). Based on a subset-analysis (data not shown) of the present data, results indicate that sensitivity of direct EPO tests are ~90%, while sensitivity is ~46% for the indirect testing methods.

Strengths and limitations

The applied dosing regimen and the likelihood of reflecting a real-world scenario must be considered when interpreting the ergogenic effects of rHuEPO. As athletes may misuse prohibited substances in a clandestine manner an accurate replication of real-world administration practices is difficult, but the dosing regimen applied resembles testimonies of former professional athletes who abused rHuEPO(23). Despite the inclusion of an equal number of participants from both sexes and utilization of a rigorous study design, potential sexually dimorphic responses may not be discovered due to the sex-specific sample size in the rHuEPO group. The inclusion of recreational to well-trained individuals have been argued to limit a direct translation to performance-enhancing effects in elite athletes, since elite athletes already are at the top tier of their performance, thus potentially less influenced by small alterations in for instance tHb. Nevertheless, an increased oxygen-carrying capacity is likely to result in superior performance of elite athletes as well, exemplified by a repetitive Olympic and World Champion with autosomal dominant erythrocytosis resulting in a hematocrit of 60-70%(56). Furthermore, apart from one female participant, the increase in tHb was independent of the initial tHb level,
which ranged from 425-1.194 g (data not shown, $r^2 = 0.00$), suggesting that the effects of rHuEPO is independent of the initial levels of oxygen-carrying capacity. This also aligns with the vast amount of evidence on this topic pointing to an actual ergogenic effect of rHuEPO(3) and the fact that no differences in e.g. pacing during a time trial seems evident between elite and untrained individuals(57). Finally, we did not control for menstrual cycle which potentially could have influenced the results. However, a recent meta-analysis concluded that performance might only be trivially reduced during the early follicular phase compared to all other menstrual phases(58), while the impact of the menstrual cycle is likely negligible.

**CONCLUSIONS**

In conclusion, 9 IU×kg bw$^{-1}$ epoetin β three times per week for four weeks provides a sufficient increase in erythropoiesis to augment tHb and increase performance quantified as a preloaded time trial and $\dot{V}O_2$peak in both male and females. Thus, continuous efforts to deter and detect rHuEPO misuse are required.
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Conflicts Of Interest

The results of the present study do not constitute endorsement by the American College of Sports Medicine. The results of the study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation. ABA is employed as a Doping Controlling Officer at Anti-Doping Denmark. ABA and TCB was funded by WADA and Team Denmark and JB by Partnership for Clean Competition. The authors have no conflicts of interest to declare.

Clinical Trial Registration

Clinical trials identifier, NCT04965961.
REFERENCES


FIGURE LEGENDS

**Figure 1.** Flow diagram (A) and study design (B). After randomization to either the placebo or recombinant human erythropoietin (rHuEPO) group, participants were familiarized twice (abbreviated FAM1 and 2) with the time trial (TT) protocol before the pre-treatment TT and peak oxygen uptake ($\dot{V}O_{2\text{peak}}$) tests was performed. Following, the four-week treatment period, TT and $\dot{V}O_{2\text{peak}}$ was performed three, and five days following last injection. Carbon monoxide (CO) rebreathing to determine total hemoglobin mass was performed before and three days after last injection.

**Figure 2.** Mean values for mean power output (MPO) and time to completion (TTC) in histograms with individual participants as lines in females (orange; n=24), males (blue; n=24) and pooled data from all (green, n=48) before (light color) and after (dark color) treatment with recombinant human erythropoietin (rHuEPO) or placebo (PLA). * indicate “time × treat” effect, $P<0.05$. ### indicate difference from pretreatment test, $P<0.001$.

**Figure 3.** Mean values for peak oxygen uptake ($\dot{V}O_{2\text{peak}}$) and incremental peak power output (IPPO) in histograms with individual participants as lines in females (orange; n=24), males (blue; n=24) and pooled data from all (green, n=48) before (light color) and after (dark color) treatment with recombinant human erythropoietin (rHuEPO) or placebo (PLA). * and ** indicate “time × treat” effect, $P<0.05$ and $P<0.01$, respectively. ## indicate difference from pretreatment test, $P<0.01$. 

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Figure 4. Mean values for total hemoglobin mass (tHb) in histograms with individual participants as lines in females (orange; n=24), males (blue; n=24) and pooled data from all (green, n=48) before (light color) and after (dark color) treatment with recombinant human erythropoietin (rHuEPO) or placebo (PLA). *** indicate “time × treat” effect, P<0.001, ### indicate difference from pretreatment, P<0.001.

Figure 5. Mean values for hematocrit in females (orange; n=24), males (blue; n=24) and all (green; n=48) for placebo (PLA; unfilled circles) and recombinant human erythropoietin (rHuEPO; filled circles) during baseline (B1 and B2), treatment (W1-4) and follow up (three, five and 10 days after last injection denoted 3D, 5D and 10D, respectively). Statistically significant differences: *** and ### above the line indicate significant “time × treatment” and “time × treatment × sex” effect, respectively, P<0.001. *, **, ***, P<0.05, P<0.01, P<0.001 indicate differences between PLA and rHuEPO at specific time points, respectively. Parentheses () indicate a statistical tendency (P<0.1).

Figure 6. Correlation between changes in total hemoglobin mass (ΔtHb) and peak oxygen uptake (A; ΔVO₂peak), mean power output (C; ΔMPO) and time to completion (E; ΔTTC) as well as correlation between changes in hematocrit (ΔHct) and ΔVO₂peak (B), ΔMPO (D) and ΔTTC (F).
Table S1 - Body composition before and after recombinant human erythropoietin (rHuEPO) treatment. Mean ± SD values for body weight, body mass index (BMI), fat mass (FM), fat free mass (FFM) and body fat percentage (BF%) *, P<0.05 difference between the two treatment groups. #, P<0.05 difference between males and females.

Table S2: Mean ± SD values for systolic (Sys) and disastolic (Dia) blood pressure during and 3, 5, and 10 days after recombinant human erythropoietin (rHuEPO) or placebo treatment. No statistical differences were found.

Table S3: Mean ± SD values for pulmonary data, heart rate (HR) and mean power output (MPO) before (PRE) and after (POST) treatment with recombinant human erythropoietin (rHuEPO) or placebo. V̇E: ventilation, V̇O₂: oxygen uptake, V̇CO₂: carbondioxide exhalation, RER: respiratory exchange ratio.

Table S4: Mean ± SD values for blood metabolites and ions before and after treatment with recombinant human erythropoietin (rHuEPO) or placebo. For clarity, any ‘time’ main effects are not denoted.
**Figure S1:** Mean values for red blood cell volume (RBCV), plasma volume (PV) and total blood volume (BV) in histograms with individual participants as lines in females (orange; n=24), males (blue; n=24) and pooled data from all (green, n=48) before (light color) and after (dark color) treatment with recombinant human erythropoietin (rHuEPO) or placebo (PLA). * and *** indicate “time × treat” effect, P<0.05 and P<0.001, respectively. # and ### indicate difference from pre-treatment, P<0.01 and P<0.001, respectively.
Table 1. Mean ± SD values for pulmonary data and exercise capacity. rHuEPO: recombinant human erythropoietin, $\dot{V}O_{2\text{peak}}$: peak oxygen uptake, rel. $\dot{V}O_{2\text{peak}}$: peak oxygen uptake relative to bodyweight, HR$_{\text{max}}$: maximal heart rate, BPM: beats per minute, IPPO: incremental peak power output. #, P<0.05 difference between males and females.

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Figure 2
Figure 3
Figure 4