Eur J Appl Physiol
DOI 10.1007/s00421-009-1259-3

SHORT COMMUNICATION

Assessment of total haemoglobin mass: can it detect erythropoietin-induced blood manipulations?

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Accepted: 12 October 2009

Abstract The purpose of the study was to reveal erythropoietin (epo) doping. It was recently suggested that the assessment of total haemoglobin mass (tHb) by means of the carbon monoxide re-breathing technique should be implemented in anti-doping work. Since epo may increase the haemoglobin concentration [Hb] simply by reducing plasma volume we injected eight human subjects with epo for 15 weeks and directly tested the feasibility hereof. Epo treatment increased [Hb] in all subjects at all time points (range 3.8–18.8%). In approximately half the subjects this was mainly the consequence of an increased tHb, but in the remaining subjects the change was the result of a decrease in the plasma volume. After the initial epo "boosting" period the assessment of tHb could not detect epo injections in 50% of the subjects in the remaining "maintenance" period. In our opinion the variability observed over time when assessing tHb is not justifiable in an anti-doping setting.

Keywords Epo · Doping · Test · Performance · Carbon monoxide

Communicated by Susan Ward.

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Introduction

Agents or procedures misused with the intent to artificially increase arterial oxygen content (CaO_2) are banned by the World Anti Doping Agency (WADA) and are considered as doping. In an attempt to reveal blood and erythropoietin (epo) doping in athletes, it was recently suggested that a modified carbon monoxide (CO) re-breathing technique (Schmidt and Prommer 2005) is used to quantify total haemoglobin mass (tHb) (Prommer et al. 2008). One clear advantage using tHb for anti-doping purposes is that unlike [Hb], the analytical result is independent to hydration status and/or plasma volume manipulations. The rationale for assessing tHb in elite athletes is that fluctuations in tHb above a certain magnitude (yet to be determined) could be indicative of doping. Although tHb can increase with training in combination with altitude exposure (Brugniaux et al. 2006), it seems that tHb is stable (Eastwood et al. 2008) and is not influenced much by brief periods of training (Green et al. 1991; Shoemaker et al. 1996). Data from longitudinal studies are scarce, but in active healthy subjects the assessment of tHb over 100 days showed variation of approximately 2% (Eastwood et al. 2008). In athletes, however, the average oscillation over 1 year is reported to be 4.6%, with the maximum individual fluctuation being 6.9% (Prommer et al. 2008). Obviously the naturally occurring oscillation should be included in the yet to be determined allowed fluctuation. While it seems safe to say that the infusion of red cells into the circulation will increase tHb (Pottgiesser et al. 2007), this does not necessarily apply to epo doping. In a recent study we demonstrated that low dosage epo treatment increased haemoglobin concentration [Hb] on average from 14.2 ± 0.6 to a peak of 17.1 ± 0.5 g dl^{-1} after 12 weeks of epo administration (Lundby et al. 2007). Quite surprisingly, this increase was the

Published online: 29 October 2009

product of (1) increased red cell volume induced by erythropoiesis and (2) a concomitant decrease in plasma volume. The mechanism by which epo increased [Hb] had great subject variability (i.e. increased erythropoiesis and/or decreased plasma volume), and in some subjects the quantification of tHb would not have revealed the fact that the subjects had been injected with epo because the increase was less than the 4.6% average oscillation reported by Prommer et al. (2008). Obviously, the results become even worse if the maximum individual fluctuation (6.9%) is used instead of the average oscillation. Here, we present the individual tHb and [Hb] data from the above mentioned study where we injected epo in human subjects and quantified tHb by the CO re-breathing technique (Lundby et al. 2007). The feasibility of implementing the CO re-breathing method to determine potential epo doping is discussed.

Methods

The study was approved by the local ethical committee of the communities of Copenhagen and Frederiksberg and conformed to the Declaration of Helsinki. All subjects gave written informed consent to participate. For full methodological description the reader is referred to (Lundby et al. 2007). To briefly summarize, human subjects were subcutaneously injected with epo (5,000 IU) frequently (3–4 times/week) during the first 3 weeks to rapidly increase [Hb] (Boosting period), where after the injection frequency was decreased to one injection per week for the next 11 weeks (Maintenance period). tHb mass was measured twice before any epo injection and then after 5, 11 and 13 weeks. tHb was determined by means of a CO-rebreathing method, based on the protocol of (Burge and Skinner 1995). Briefly, after the subject had stayed for 20 min in a semirecumbent position, a 2.0-ml blood sample was obtained from an antecubital vein for immediate determination of carboxyhaemoglobin (%HbCO) via a 20G catheter. The subject then breathed 100% O_2 for 4 min to flush nitrogen from airways, before being switched to the re-breathing circuit. A bolus of 99 ml of CO (99.997% chemically pure, Linde gases, Pullach, Germany) was then added to the circuit and the subject re-breathed for 10 min. At minute 10, just before the subject was disconnected, a second blood sample was withdrawn. Average % HbCO among our subjects was 1.6% at baseline and reached 8.2% after CO inhalation. %HbCO was not corrected for oxygen saturation. Carboxyhaemoglobin and [Hb] were analysed in triplicate for each measurement on an automated system (Radiometer ABL700; Radiometer, Copenhagen, Denmark). Here the per cent typical errors (Hopkins 2000) calculated from duplicate baseline measurements were 2.4% for tHb and 1.6% for [Hb].

Results

Erythropoietin treatment increased [Hb] in all subjects at all time points as compared to baseline measurements (range 3.8–18.8%) (Table 1). After 5 weeks of epo injections tHb was increased in seven out of eight subjects (range 9.4–13.9%), but only by 1.3% in one subject (Table 1; Fig. 1). After 11 weeks of epo treatment tHb (as compared to baseline values) was altered by <4.6% in four subjects (−0.9 to 3.3%) and by more than 4.6% in the remaining

Table 1 Haemoglobin concentration and total haemoglobin mass in epo treated subjects

Subject	Haemoglobin concentration (g l^{-1})				Total haemoglobin mass (g)				Total haemoglobin mass (g kg^{-1})			
	Before	5 Wk	11 Wk	13 Wk	Before	5 Wk	11 Wk	13 Wk	Before	5 Wk	11 Wk	13 Wk
#1	149	164	154	156	981	1,102	1,014	1,019	12.71	14.35	13.10	13.10
#2	122	143	135	130	871	992	863	896	10.82	12.22	10.65	10.94
#3	151	169	172	164	977	1,074	1,079	1,069	12.30	13.41	13.40	13.36
#4	152	174	180	167	1,087	1,188	1,119	1,119	13.10	14.40	13.47	13.32
#5	147	156	162	154	778	855	783	808	10.56	11.71	10.70	10.96
#6	148	160	165	171	891	985	966	987	9.89	11.01	10.51	10.65
#7	150	163	166	163	884	987	943	964	11.13	12.49	11.64	11.83
#8	150	166	171	171	1,188	1,204	1,378	1,425	12.68	12.81	14.63	15.00
Mean	146	162*	163*	159*	957	1,049*	1,018*	1,036*	11.65	12.80*	12.26*	12.40*
SD	10	9	14	13	131	117	182	185	1.19	1.21	1.58	1.54

Individual values, means and standard deviations for eight subjects. Baseline values correspond to the average of two determinations made on separate days

* $P < 0.05$ versus before epo treatment

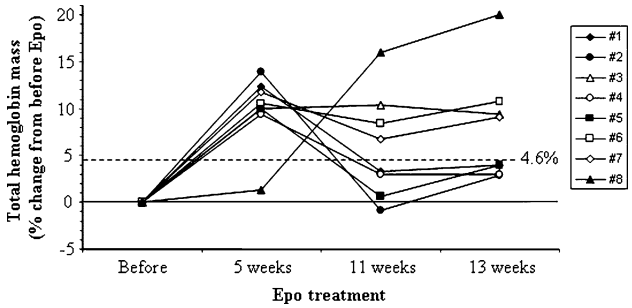

Fig. 1 Total haemoglobin mass after 5, 11 and 13 weeks of rhEpo, expressed in percentage change from baseline. The *upper dotted line* indicates the average oscillation of 4.6% previously reported (Prommer et al. 2008)

four subjects (6.8–16.0%). After 13 weeks of epo treatment tHb was increased (compared to baseline) in four subjects by <4.6% (2.9–3.9%), and by more than 4.6% in the remaining four subjects (9.1–20.0%). In those subjects where tHb was only changed modestly, decreases in plasma volume effectively increased [Hb]. As we have reported elsewhere VO_2max and exercise performance were enhanced in all subjects at all investigated time points (Lundby et al. 2008a; Thomsen et al. 2007).

Discussion

Assuming 4.6% as average maximal oscillation over a year in tHb of athletes (Prommer et al. 2008) we found the CO re-breathing method to have the potential for a high detection rate in the early phase of epo doping (5 weeks), but that the detection rate is drastically decreased over time, and at time points 11 and 13 weeks, the method had a success rate of just 50%.

Because the window of opportunity to determine epo doping is very short (Lundby et al. 2008b) the introduction of the so-called hematological passport (Cazzola 2000) seems more timely than ever. This approach proposes to allow athletes a certain degree of fluctuation in blood markers such as [Hb], haematocrit and reticulocytes, and if preset "normal" values are surpassed by a given magnitude, this could be indicative of doping, and sanctions could be employed. Recently it was suggested to include the assessment of tHb in this passport. We are concerned with this approach because we found that the recently published average oscillation in tHb over 1 year in athletes (Prommer et al. 2008) surpasses the increase in tHb that may be achieved by epo doping. Indeed, our finding indicating that epo injections increased tHb by <4.6% in 50% of the subjects after 11 and 13 weeks of treatment demonstrates that these subjects would not be determined as "dopers" if investigated by the CO re-breathing method. An epo-induced increase in tHb of less than the average oscillation of 4.6% observed by Prommer and co-workers may obviously still increase exercise performance (Lundby et al. 2008a; Thomsen et al. 2007; Calbet et al. 2006). It should also be noted that the values for other markers proposed for inclusion in the blood passport such as reticulocytes were, at these time points, not different from those obtained prior to epo injection (Lundby et al. 2007), and hence also do not reveal the fact the subjects were injected with epo. Although this may seem disappointing, the introduction of the blood passport in our opinion is presumably a better solution than relying on the current WADA urine based epo test because of its low detection rates (Lundby et al. 2008b).

In our opinion, the variability observed over time when employing the CO re-breathing method is not justifiable in an anti-doping setting aiming to detect epo doping, and not much is gained in comparison to presently assessment of [Hb]. In addition, it is seen in Table 1 that [Hb] is increased in all subjects at all time points when injected with epo. Also, despite that the CO re-breathing procedure is simple, straight forward and can be performed without great physiological/technical knowledge, it is far more logistically demanding and the implementation of the method in field conditions seems very ambitious. Furthermore, while [Hb] assessments rely on automated and validated clinical chemistry, the between-laboratory reproducibility of tHb assessment seems another major obstacle that has to be overcome. Also, CO re-breathing requires full subject collaboration and not all subject cope with the re-breathing procedure. This being said, however, the assessment of [Hb] is not without concerns either, and besides physiological variation (Thirup 2003), factors such as pre-analytical variability (e.g. alterations in posture, ambient temperature, hydration and/or manipulations with saline solutions) and analytical reproducibility may all contribute to the total error during [Hb] determination.

The fact that we did not assess tHb with the modified CO re-breathing method proposed to be implemented in anti-doping settings (Prommer et al. 2008; Schmidt and Prommer 2005) but with classic procedure as developed by Burge and Skinner (Burge and Skinner 1995) has to be considered. First, re-breathing time is substantially different between the two approaches (10 min for Burge and Skinner (1995) vs. 2 min for Schmidt and Prommer (2005)), thus raising the question of the comparability of the results. However, recent evidence shows that both procedures yield similar %HbCO values (and therefore tHb), provided that sampling is performed after that the blood CO concentration has stabilized (Gore et al. 2006). Secondly, blood sampling site (capillary for Schmidt and Prommer's vs. venous for Burge and Skinner's technique) may induce additional variation since %HbCO is known to be altered by oxygen

saturation (Hütler et al. 2001). Since we did not correct for the lower oxygen saturations in venous blood, we cannot exclude that this has not introduced additional variability to our measurements.

Typical errors obtained from reliability studies, using either the Burge and Skinner's technique (0.8% in Burge and Skinner 1995; 1.9% in Eastwood et al. 2008) or the modified CO re-breathing technique (1.7% in Schmidt and Prommer 2005; 1.1% in Gore et al. 2006), indicate that both techniques are equivalent in term of precision. We are therefore confident that our analysis, based on the Burge and Skinner's technique, is also applicable to the modified CO re-breathing method. We acknowledge that our typical error for tHb of 2.4%, is not as low as those reported in previous studies, but nevertheless conforms to the overall measurement error of 2.2% associated with the CO re-breathing method (Gore et al. 2005).

Our conclusion is that the assessment of tHb mass by any given CO-rebreathing technique is not suitable for detecting low dose epo doping.

Acknowledgments This study was financially supported by grants from Anti Doping Denmark.

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