Testing for recombinant human erythropoietin in urine:
problems associated with current anti doping testing.


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Abstract

Background: The main action of recombinant human erythropoietin (rHuEpo) is to increase the oxygen carrying capacity of the blood. To prevent a possible misuse of rHuEpo, this is tested in urine samples collected from athletes by World Anti Doping Agency (WADA) accredited laboratories. Recently the test has met serious critiques, and the aims of the present study were to investigate the detection power of the test as well as the variability in the test power comparing the results of two WADA accredited laboratories.

Methods: Eight human subjects were studied for seven weeks and treated with rHuEpo for four weeks with two weeks of “boosting” followed by two weeks of “maintenance” and a post period of three weeks. Urine samples were obtained during all periods.

Results: Laboratory A determined rHuEpo misuse in all subjects during the “boosting” period, whereas Laboratory B found no misuse, with one sample to be negative, and the remaining seven to be suspicious. The detection rates decreased throughout the maintenance and post period when total hemoglobin mass and exercise performance were elevated. During this period, laboratory A found only two out of 24 samples to be positive, and three to be suspicious, and laboratory B found no positive or suspicious samples.

Conclusion: This study demonstrates a poor agreement in test results comparing two WADA accredited laboratories. Moreover, after the initial rHuEpo “boosting” period the power to detect rHuEpo misuse during the maintenance and post periods appears minimal.
Introduction

Erythropoietin (Epo) is a glycoprotein hormone that is mainly produced in the kidney (7) and enhances the oxygen carrying capacity of the blood by increasing haemoglobin mass and concomitantly decreasing plasma volume (14). Injections with recombinant human Epo (rHuEpo) greatly increases aerobic performance in humans (6; 17), and although the majority of the increase in performance is associated to oxygen transport, also non-Hb mediated functions of rHuEpo may be speculated to increase performance (see (13) for references). rHuEpo is banned for performance enhancement by the World Anti-Doping Code. Accredited World Anti Doping Agency (WADA) laboratories test for potential rHuEpo doping using a method developed by the research group of Lasne (10). The test is based on the assumption that endogenous and rHuEpo’s separate according to their charge heterogeneity (18; 19), and Epo isoelectric focusing is the official method used on a routine basis in all WADA accredited anti-doping laboratories. The method has met serious critiques, with cases of false positive testing in an experimental setting (2) (albeit the findings hereof has been disputed by others (4; 11)) and very low detection power (9). In addition, the half life of rHuEpo is rather short, making detection difficult three days after injection (15), and a case report has even implied that detection may be impossible after as little as 12-18 hours after injection (1). Also the so-called ON-model has been criticized (12), and other potential pitfalls of urine rHuEpo testing has recently been reviewed by Delanghe and co-workers (5).

In the present study rHuEpo was injected in eight human subjects for four weeks to study haematological effects of rHuEpo. At the same time we took the opportunity to assess performance and the detection power of the WADA test, as well as to test the variability of rHuEpo urine analysis in two different accredited laboratories.
Methods

Eight healthy male (23 ± 3 yr, 181 ± 7 cm, 77 ± 5 kg) volunteers (university students, non athletes) participated in the study. 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.

5,000 IU rHuEpo (NeoRecormon, Roche, Mannheim, Germany) was injected as follows: The first 14 days (“boosting” period) subjects were injected with rHuEpo every second day, and the following two weeks a single injection was given weekly (“maintenance” period). Prior to the injection base line measurements were obtained (repeated twice). Furthermore, blood samples from an arm vein were obtained prior to rHuEpo injections and on days 14, 16, 21, 23, 28, 35, 42 and 49. Urine samples were collected on days 0, 16, 23, 30, 35, 42, and 49 before any blood sampling where also total hemoglobin mass was determined. Exercise tests were conducted prior to rHuEpo treatment and on days 35, 42, and 49.

The venous blood samples were analyzed for hematocrit and total plasma Epo concentration (Quantikine, R&D Systems, Minneapolis, Minnesota, USA). Total haemoglobin mass was determined by the carbon monoxide re-breathing method (3). Urine samples were obtained under sterile conditions with visual inspection by an experimenter. Each urine sample was handled according to WADA rules and immediately divided into four or five containers and stored at -80 ºC. The samples were subsequently shipped to laboratories for later analysis packed in dry ice (78.5 ºC or colder) and were reported by the laboratories to have arrived packed in dry ice and still to be in the solid frozen state. Total transport time was less than 24 hours for both laboratories. Identical urine samples were analyzed in duplicates according to WADA regulations (http://www.wada-ama.org/rtecontent/document/td2004epo_en.pdf) by two independent WADA laboratories which
were blinded toward the treatment. The results were categorized by these WADA accredited labs as “negative”, “suspicious”, or “positive”. Only samples determined “positive” can be used to potentially ban an athlete from competition, whereas samples found “suspicious” and “negative” do not imply restrictions. In “real life” WADA requires labs that confirm an adverse analytical finding for rHuEPO to ask another lab to repeat the analysis. This was not done in the present study.

The exercise were performed using a bicycle ergometer with concomitant determination of pulmonary gas exchange as described previously (17).

Statistical differences over time were assessed by the non parametric Friedman test, and the non parametric Wilcoxon test was used as post hoc test. Statistical difference was set to p<0.05. All values reported are mean±SD.

Results

The rHuEpo treatment was effective in increasing total Hb mass at all measuring points (Table 1). Similarly, the hematocrit was augmented except on Day 49 where it had returned to base values. Aerobic exercise power was increased (5.4 – 7.9 %; p = 0.034) during the performance tests on days 35, 42 and 49. The urine data are shown in Table 1.

Discussion

Laboratory A determined rHuEpo misuse in all subjects during the “boosting” period, whereas Laboratory B found no tests to be judged as positive. In the “maintenance” period laboratory A found six positive results indicating misuse and two suspicious results in a total of 16 samples. In the same period laboratory B found five samples to be suspicious. During the post treatment period laboratory A found two out of 24 samples to be positive, and three to be suspicious. Laboratory B found all these samples to be negative. There was little consistency in results between Laboratories
A and B, with a suspicious sample from laboratory A not being confirmed as a suspicious or positive sample from laboratory B, and vice versa.

The results can be interpreted as laboratory A having a higher positive determination rate as compared to laboratory B. In the boosting period this can not be attributed to false positives since the subjects were injected with rHuEpo. Although this would seem satisfying for Lab A, it may be of some concern that this lab found a sample to be positive on Day 35 when the same person was negative on day 30. This is of concern because no injection had occurred between the two measurement points. In addition, the fact that two samples were determined positive on day 35 from Lab A suggests that either the detection window is wider than the previous reported three days (15) in some subjects, or they are false positives. Regardless, it is of main concern that the correlation between test results from Lab A and B are poor, and that Lab B was not able to determine a single sample as being positive. The origin of the differences in analytical outcome between the laboratories is unknown (and beyond the scope of the present study), but could include interlaboratory standardization difficulties because of the use of irreproducible carrier ampholyte gels (16). Also, the highly concentrated urine sample is difficult to solubilise and apply on the gel (8). Although highly concentrated, some 20% of analysed urine samples do not show detectable epo (16) suggesting low test sensitivity. The American WADA laboratory has analysed over 2600 urine samples for rHuEpo, and hereof 9 have been found positive. This low detection rate could be interpreted as either athletes are not misusing rHuEpo, or that the testing for short acting peptide hormones in urine is difficult.

During the maintenance period and three weeks after the last rHuEpo injection, when total hemoglobin mass and aerobic exercise capacity were still elevated, only two out of 48 samples were found positive. Moreover, it is worth noting that only two out of 16 samples were found positive on
day 30 (i.e. 48h following previous injection), whereas this time point lies within the detection time window of three days (15). This implies that athletes may take advantage of rHuEpo doping without great risk of being tested Epo positive. It is noteworthy that a maintenance dose of rHuEpo, which in this study was limited to two weeks, can by the athlete be elongated throughout a whole season (14). The practical implication is then that urine testing during competition in the season is of little or no value. The only strategy which provides a possible chance for obtaining a positive urine Epo test is to find the athlete for out of competition testing during a “boosting” period.

In conclusion, it would seem that for urine determinations of rHuEpo to be as efficient as can be with present laboratory techniques, elite athletes should be tested out of competition at strategic time points in their preparation for a season or for major events. The obtained samples would have to be analysed by more than one laboratory. On the other hand, it should be considered whether it is at all advisable to spend more energy and money on the present procedures or developing new rHuEpo tests considering the availability of pharmacological agents such as continuous erythropoietin receptor activators (CERA) and Hydrol Proxylases having essentially the same effect as rHuEpo without the necessity of (mis)using rHuEpo. The potential implementation of the blood passport where longitudinal monitoring will be used to identify changes indicative of doping practices seems to surpass many of the above problems.
Table 1. Total hemoglobin mass (Hb mass, g), hematocrit (Htc, %), Maximal pulmonary oxygen uptake (VO₂max, ml.min⁻¹), and number of samples determined “positive” from laboratory A and B, respectively obtained before any rHuEpo injection (Pre rHuEpo), during (rHuEpo treatment period), and after (Post rHuEpo treatment period) rHuEpo injections. p values indicate the level of significance as compared to Pre rHuEpo treatment.

<table>
<thead>
<tr>
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<th>Pre rHuEpo</th>
<th>rHuEpo treatment period</th>
<th>Post rHuEpo treatment period</th>
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<tr>
<td></td>
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<td>Day 14 (2 days later for urine and serum epo)</td>
<td>Day 21 (2 days later for urine and serum epo)</td>
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<tr>
<td>Hb mass, g</td>
<td>922 ± 96</td>
<td>1,002 ± 112 p=0.012</td>
<td>1,016 ± 144 p=0.012</td>
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<td>Htc, %</td>
<td>43.9 ± 1.8</td>
<td>46.1 ± 2.2 p=0.012</td>
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<td>Serum Epo, ng.l⁻¹</td>
<td>78 ± 41</td>
<td>226 ± 76 p=0.012</td>
<td>90 ± 60 p=0.484</td>
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<td>VO₂max, ml.min⁻¹</td>
<td>3,949 ± 561</td>
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<td>Number of urine sample tested</td>
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<tr>
<td>Laboratory A: Positives for rHuEpo</td>
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<td>Laboratory B: Positives for rHuEpo</td>
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