

PROJECT REVIEW

“Detection of rEPO Abuse in Athletes”

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In order to improve their performance, some athletes utilize methods which optimize the physiological characteristics needed for their sport. The considerable availability of recombinant human erythropoietin (rEPO) has allowed the widespread use of this drug in aerobic sports to increase oxygen transfer capacity. Like endogenous EPO the recombinant hormone interacts with the precursor erythroid cells causing proliferation and differentiation of these cells in mature erythrocytes (1). Although rEPO has been banned by the medical commission of the International Olympic Committee, the anti-doping tests currently available cannot detect it with confidence. A direct detection of rEPO in urine has recently been suggested (2).

However, while the plasma half-life of rEPO varies between 4 and 13 hours (3) its biological effects occur several days after treatments and thus the erythropoietic effect becomes evident when rEPO is no longer detectable in circulation. Moreover, EPO concentrations are not only very low but also vary considerably from one person to another and are influenced by environmental factors such as fatigue, stress and body hydration. In order to overcome these limitations and make the abuse of rEPO detectable, Gareau *et al.* (4) and subsequently Bressoll *et al.* (5) suggested that, besides the hematocrit value, the ratio between the concentration of the soluble transferrin receptor (a marker of erythroid activity) and the concentration of serum ferritin (a measure of the iron stored in the body) should also be evaluated. In our previous study (7) we evaluated the concentration of the soluble transferrin receptor (sTfr) and the concentration of serum ferritin (fr), to detect the abuse of rEPO as suggested by Gareau *et al.* (4). In groups of athletes treated utilizing different protocols for rEPO, iron, folic acid and vitamin B₁₂ administration, we demonstrated that the sTfr/fr ratio depends on the administration schedule and can vary upon iron supplementation. Our results confirm the conclusion of Gareau only when rEPO is administered at high doses and without iron supplementation. In contrast, when rEPO is administered at low doses and associated with an iron

supplement, as is common in clinical practice to obtain a significant and lasting hematocrit increase (8), the sTfr/fr ratio cannot be considered a reliable marker of rEPO abuse (7). The main indirect method currently available for the detection of rEPO abuse (9) utilizes simultaneously multiple indirect hematological and biochemical markers. This method is potentially effective to identify users of rEPO but it doesn't exclude the possibility of registering false positives. Moreover, in this method there are no appropriate internal standards for inter-assay calibration and quality control procedures. In our laboratory we evaluated the effects of different rEPO administration protocols on the levels of β -globin mRNA, a selective marker of erythroid activity expressed during erythropoiesis stimulation, and Tfr mRNA, detected by RT-PCR. We found that the amounts of β -globin and Tfr mRNAs in

whole blood significantly increase in all treatments investigated. Prompted by these results, we developed a mathematical equation that takes into account hematological, biochemical and molecular values that changed most significantly during treatment. The values considered were hematocrit (Ht), reticulocyte count (Ret), sTfr, Tfr mRNA and f3-globin mRNA and allowed the detection of rEPO abuse regardless of the administration schedule and iron supplement. In our multiparametric equation the values that play a critical role are f3-globin and Tfr mRNAs.

For J3-globin a quantitative competitive RT-PCR assay was developed; in fact accurate quantitation of nucleic acids by RT-PCR needs a valid internal standard and an adequate mathematical model for data analysis. To address these points we first generated an internal standard, referred to as the "competitor", with a sequence very similar to that of the target amplified fragment, natural β -3-globin cDNA, but with a different size due to a 29-bp deletion. We also evaluated that the competitor and natural target f3-globin cDNA have a similar amplification efficiency, that is a crucial point for an adequate quantitative competitive RT-PCR assay.

The present proposal aims at:

- a. validating the method developed in a great number of athletes; these goals will be reached by means of further population studies. In particular the selected biological markers will be evaluated in non-professional athletes with regard to the sport practiced, gender, race, intake of iron, vitamins and other supplements;
- b. possibly reducing the number of determinations to be performed on blood samples in order to simplify the procedure, reduce the cost and time required, and provide definitive conclusions without decreasing statistical significance;
- c. generating an internal standard for Tfr mRNA in order to assure accurate quantitation by competitive RT-PCR and inter-laboratory calibration, as just performed for f3-globin;
- d. automating as much as possible the molecular procedures employed in order to reduce the time required;
- e. identifying blood denaturing solutions that allow stability and delivery of RNAs at room temperature;
- f. developing computer softwares allowing a rapid evaluation of the results obtained and thus a rapid detection of rEPO abuse.

Finally, the acceptability of this procedure for athletes will be favoured by the very small amount of blood needed to perform all determinations. We expect to validate a procedure able to detect rEPO abuse with a probability higher than 0.999999 that can be applied in different laboratories around the world.

Detection of rEPO abuse in athletes

Results and conclusions

The considerable availability of substance such as EPO has allowed the widespread use of this drug in aerobic sports to increase oxygen transfer capacity. Like endogenous EPO the recombinant hormone interacts with the precursor erythroid cells causing proliferation and differentiation of these cells in mature erythrocytes.

Although rEPO has been banned by the medical commission of the International Olympic Committee, the anti-doping tests currently used cannot detect it with confidence because of the short half-life of rEPO in plasma (4 and 13 hours), while its biological effects occur several days after treatments. We have previously developed a new indirect method based on the use of a multiparametric formula which utilizes simultaneously multiple hematological, biochemical and molecular markers changing significantly after rEPO administration. The values considered are hematocrit (Ht), reticulocyte count (Ret), soluble Transferrin receptor (sTfr-R) and β -globin mRNA (Magnani et al., Identification of blood erythroid markers useful in revealing erythropoietin abuse in athletes, *Blood Cells Mol Dis.* 2001 27:559-71. In our formula the beta-globin mRNA parameter plays a very important role since it is heavily influenced by Epo administration.

During these two years, we have optimized a procedure to collect and store the blood samples in order to assure beta-globin mRNA stability for a long term before analyses. Moreover, standardized conditions were established for determination of beta-globin mRNA in a blood sample. Subsequently the method was validated in non professional athletes receiving different forms of recombinant-Epo (Eprex® and Aranesp®) and with different protocols. Based on our previous experience, the amount of beta-globin mRNA was considered together with several other parameters including the hematocrit, reticulocyte percentage and plasma soluble transferrin receptor. These parameters were included in a multiparametric formula that provides a value for the detection for Epo abuse. The results obtained suggest that the method we have developed can be conveniently used in a large interval of days, but is also dependent upon Epo administration regime, sex and Epo molecular form.