In: Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (eds.)
Recent Advances in Doping Analysis (1). Sport und Buch Strauß, Köln 1994

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Induced haemostatic shift: a possible tool for EPO detection[1].

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Introduction

Basic physiology teaches that maintenance of physiological processes into homeostatic boundaries is a prerequisite condition to the integrity of living organisms. For elite athletes, maximal performances are reached by stretching these boundaries to their extreme limits that is, maintaining a functional "milieu intérieur" (dixit Claude Bernard) in spite of conditions considered adverse for most individuals. When training-induced stretching of body equilibrium is no longer possible or to cumbersome to achieve, certain athletes make appeal to ergogenic drugs.

With the advent of engineered production of erythropoietin (EPO) molecules by recombinant techniques, its clinical use rapidly showed remarkable effects in the treatment of anaemia associated with renal insufficiency. But, such an EPO-induced improvement in oxygen delivery to tissue has rapidly been recuperated by some endurance athletes. The ban promulgated on this substance by the IOC has not prevented an increasing population of athletes to seek in this hormone a practical, efficient and apparently non dangerous drug to improve their physical performance. This rapidly spreading illicit use of EPO is thus forcing the development of new detection probes by IOC-accreditated laboratories.

The apparent perfect homology of EPO with its natural counterpart, its short plasma half-life, and the delayed clinical manifestation of its effects has made this engineered molecule a doping agent of choice and a real challenge to antidoping laboratories. Our approach is based on reports that EPO could possibly influence blood homeostasis and, more specifically, the haemostatic equilibrium (Figure 1).

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[1] Results excerpted from *Gareau et al.* in Thrombosis & Haemostasis 68: 481 (1992) and in Thrombosis & Haemostasis 69: in press (1993).

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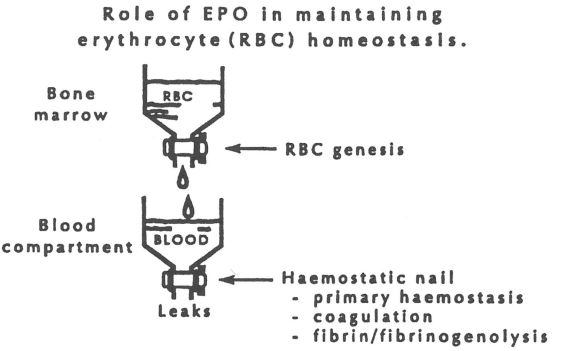

Figure 1. Schematic representation of some of the effects of erythropoietin (EPO) on the formation of red blood cells (RBC) by bone marrow and on the formation and removal of haemostatic nails.

Endogenous as well as exogenous EPO molecules are reputed to act on several processes responsible for maintaining blood homeostasis (Figure 1). Indeed, EPO can influence 1) the synthesis of erythrocytes (RBC) and 2) the formation of an haemotatic nail and its later removal by fibrinolytic and fibrinogenolytic processi. Data presented below deal mainly with the latters; indeed, we intend to demonstrate that an EPO administration could be detected by measurement of changes occuring in the lytic processi responsible for removal of hemostatic nails and/or potential clothing material floating in the blood compartment.

Before doing so, it is to be mentioned that preliminary data obtained by our group have indicated that EPO-induced erythrocyte formation is associated with the appearance in the blood compartment of increased amount of transferrin soluble receptors (Figure 2).

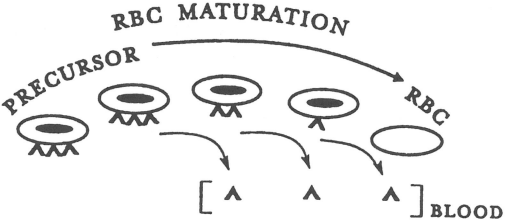

Figure 2. Schematic representation of release of transferrin receptors into the blood compartment with maturation of erythroid cells in bone marrow.

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The stress imposed by EPO on the erythroid line and accelerating the formation of reticulocytes forces the synthesis of haemoglobin molecules inside these cells. To fulfil this mandate, young cells of the erythroid line are equipped with receptors specialized in the tranfer of iron ions from circulating transferrin molecules to the porphyrin moiety of the intra-erythroblast forming haemoglobin molecules. As haemoglobin is formed, these receptors are no longer necessary and are released and solubilized into the bloodstream where they can be measured. The significant increase in the number of these soluble transferrin receptors in the blood of patients given EPO (results not shown) indicates that this variable could possibly served as a useful probe to detect EPO misusers.

EPO-induced fibrinolysis

As mentioned above (Figure 1), EPO can also influence the removal of haemostatic nails and/or circulating potential clothing material. We here describe the preliminary results obtained using two protocols, a first one with rabbits given EPO, a second one with haemodialized patients receiving or not anti-anemia EPO treatments.

- *Animal studies*

A first protocol comprised twelve male and female rabbits of different chronological ages that were randomly distributed into three groups. Group A animals (6 rabbits) served as controls. Group B rabbits (3 animals) were administered 250 IU/kg of EPO subcutaneously for 4 consecutive days. The 3 animals in group C were given 500 IU/kg of EPO but on day 0 and on day 2, only. Blood samples were collected on day 0 prior to EPO administration, and thereafter on days 8, 15 and 21. Haematologic (Hct, RBC and RBC indices) and coagulation (PT, APTT, TT, protein C antigen, AT III, vWF activity and D-Dimer quantification) parameters were examined on each blood sample. Each analysis was performed according to manufacturer's specifications. Mouse-raised monoclonal antibodies were used in our analytical systems for vWF and D-Dimer measurements.

- *D-dimer and von Willebrand*

Visual examination revealed no untoward effects in either group of rabbits treated with EPO. Table 1 summarizes our observations. As expected significant variations were observed in RBC and Hct. A dose-dependent effect of EPO on the level of activity of the von Willebrand factor and on the D-Dimer quantification was observed. Indeed, marked increases in von Willebrand factor activity level (30 and 60%) and D-Dimer quantification (100 and 200%) were observed with the 250 and 500 IU/kg doses, respectively.

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Administered EPO (IU/kg)	Sampling (day)	RBC ($x10^{12}$l)	Hct (%)	vWF (%)	D-Dimer (ng/ml)
Group A	0	5.24±0.01	0.34±0.002	13.5±0.9	<500
(0)	8	5.22±0.02	0.33±0.004	17.5±0.5	<500
	15	5.11±0.02	0.33±0.002	12.0±0.7	<500
	21	5.16±0.03	0.34±0.003	16.5±0.6	<500
Group B	0	5.15±0.02	0.33±0.003	19.2±0.6	<500
(250)	8	5.47±0.02	0.38±0.003	39.2±1.6	>1000
	15	5.57±0.03	0.38±0.002	48.0±1.0	>1000
	21	5.33±0.03	0.33±0.003	15.0±1.2	>1000
Group C	0	5.91±0.02	0.37±0.002	15.8±1.5	<500
(500)	8	6.29±0.02	0.42±0.003	85.1±1.7	>1000
	15	6.23±0.03	0.41±0.002	80.9±2.9	>2000
	21	---	---	20.3±1.2	>2000

Table 1. Mean (± SEM) values for hematologic parameters measured repeatedly in rabbits treated with different EPO dosages. Values in squares are markedly different from control values (Group A). Because of the small number of animals in each group the discriminating power of ANOVA does not permit further statistical precision.

Most interestingly in a drug-abuse detection perspective is the fact that - EPO-induced variations were still noticeable 21 days after treatment. It appeared also that these induced effects were not related to the sex or the age of the animals.

The von Willebrand factor, involved in the initiation of haemostatic nail formation, is a giant glycoprotein (1 - 12 x 10^6 kDa) with a half-life of about 18 h and is produced by megakaryocytes and vascular endothelial cells. It is an important factor in primary haemostasis and is closely related to platelet adhesion. Decreases in vWF activity have been well documented, especially in vW disease where a decrease in quantity or in the state of polymerization is most often observed. Some authors reported no significant change in the level of activity of vWF in hemodialyzed patients studied before and 1, 4 and 8 weeks after commencing EPO. These observations were not supported by others reporting an increase in the concentration of vWF in hemodialysed patients with renal anemia receiving EPO. In a sports medicine perspective no physiological condition, to our knowledge, is associated with an increase in the vWF activity. We also described a marked increase in the concentration of D-Dimer in rabbits treated with EPO. It is well known that D-Dimer quantification is a good indicator of an acceleration of fibrinolysis.

These observations suggested that administration of EPO to rabbits induced an activation of primary haemostasis and fibrinolysis. Persistence of these EPO-induced effects after several weeks is most interesting. Further studies were needed to evaluate in humans the relevance of these observations. The second study evaluates the effect of rHuEPO on the content of urinary fibrinolytic products in human subjects.

Human studies

The recent development of sensitive immunoassays for fibrin/fibrinogen degradation products allow for a better identification of the degradation pathways involved (fibrinolysis or fibrinogenolysis).

Thirty-six male and female subjects of different ages were distributed into three groups: group A comprised 22 healthy control subjects; group B included 7 haemodialysed patients who never received EPO (EPREX, Ortho), while 7 haemo-

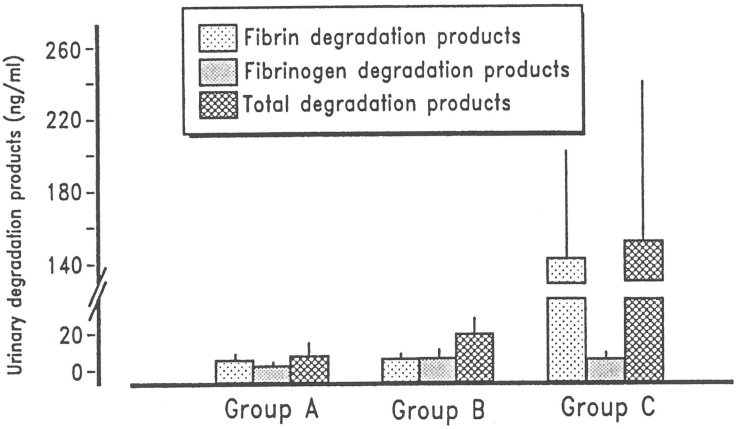

Figure 3. Mean (± SEM) urinary values (ng/ml) for fibrin, fibrinogen and total degradation products in control (group A; N = 22) and haemodialysed patients receiving (group C; N = 7) or not (group B; N = 7) recombinant human erythropoietin (EPO).

dialyzed patients treated with EPO accounted for group C. Urine samples were collected before haemodialysis for patients of group B and C, and at any time of the day for the control group (group A). In this study, fibrinolytic and fibrinogenolytic activities were preferably assessed by new sensitive immunoassays for total (TDP), fibrin (FbDP) and fibrinogen (FgDP) degradation products, using a commercial kit (Organon Tecknika).

- *Fibrinolysis*

As depicted (Table 2), renal insufficiency *per se* does not appear as a important factor in the excretion of FbDP and FgDP since no marked difference was observed for their urine content between healthy subjects of group A and untreated hemodialysed group B patients. Most interestingly, significant differences ($p < 0.001$) in FbDP (and TDP) were observed between non-treated (group B) and EPO-treated (group C) haemodialyzed patients. Moreover, these quantifications are totally independent of the urine pH known for their large fluctuations in this kind of pathology. It was also found that these results are not related to subjects' age and gender.

Our observations in human are in agreement with the activation of haemostasis and fibrinolysis observed in the blood of rabbits given EPO and extend its assessment to human urine specimens.

Conclusion

In a sports medicine context, the presence of increased amounts of FbDP in urine specimens could become a valuable probe to detect the illicit use of endurance-improving erythropoietin molecules by cheating athletes.