Features of Blood Doping

Merkmale von Blutdoping

Summary

- **Blood doping** means the use of certain techniques and/or substances to increase red blood cell (RBC) mass, which allows the body to transport more O₂ to muscles and therefore increase performance.
- **Artificial O₂ carriers** do not appear relevant in sports. Infused allogeneic blood is detectable, due to different blood groups, whereas re-transfused autologous blood is not. Recombinant human erythropoietin (rhEpo) and its analogs can be detected by isoelectric focusing and immunoblotting. There are peptidic Epo-mimetics, but none of these is clinically approved. The expression of the Epo gene (EPO) is stimulated by hypoxia-inducible transcription factors (HIFs), which consist of α- and β-subunits.
- **Hydroxylation** of the O₂-labile α-subunits can be prevented by the oral intake of cobaltous (Co²⁺) salts or α-ketoglutarate competitors (“HIF-stabilizers”). Also conceivable is the misuse of inhibitors of the GATA binding transcription factors in order to activate the EPO promoter. EPO transfer is probably not widely spread in sports.
- **The World Anti-Doping Agency (WADA)** has introduced the “Athlete Biological Passport (ABP) Operating Guidelines” for individual and longitudinal monitoring of several erythrocyte parameters (e.g., hemoglobin concentration [Hb], hematocrit in 2009. RBC count, reticulocyte numbers [Ret], mean corpuscular RBC volume [MCV] and mean corpuscular Hb mass [MCH]). Primarily [Hb] and OFF-hr score ([Hb]-60√Ret%, normal range: 85-95) are relevant with regard to sanctioning. The merit of the ABP approach is still under investigation.

**SCHLÜSSELWÖRTER:** Biologischer Blutpass, Erythropoetin, Hämoglobin, Hypoxie-induzierbare Transkriptionsfaktoren, Recombinante DNA-Technologie

Introduction

The total mass of hemoglobin (Hb mass) correlates with the rate of maximal O₂ uptake (VO₂ max) (reviewed in (23)). Red blood cell (RBC) transfusion maneuvers increase VO₂ max and prolong the time to exhaustion in the course of heavy exercise. Recombinant human erythropoietin (rhEpo) and other erythropoiesis stimulating agents (ESAs) are also misused to increase the total number of RBCs. Böning et al. (9) have discussed additional factors that could explain the improved performance after ESA doping, namely augmented diffusion capacity for O₂ in lungs and tissues, increased percentage of young red cells and da-mit die körperliche Leistung verbessert wird.


However, the authors have also noted that the importance of placebo effects must be considered since doubleblind studies are rare (9). In fact, Lundby and Olsen (35) have reasoned that there is no convincing evidence that ESAs increase exercise performance above placebo's effects other than by increasing Hb mass.

Several paragraphs of the “2016 Prohibited List” of the World Anti-Doping Agency (WADA) refer to blood doping (58). Both blood removal and reinfusion and using plasma volume expanders are prohibited. Under “Prohibited Substances” (“S2”) various ESAs are itemized: Epo, darbepoetin, methoxy polyethylene glycol-epoetin beta, Epo-mimetics, non-erythropoietic Epo receptor agonists and hypoxia-inducible factor (HIF) stabilizers and activators. Under “Prohibited Methods” forbidden blood products (“M1”), artificial O2 carriers and Hb products are described. In addition, gene doping (“M3”) is specified, including the transfer of nucleic acids or the use of normal or genetically modified cells. Note that it is not prohibited to increase Hb mass by training at altitude or in rooms with reduced O2 partial pressure. The present article provides a brief overview with respect to the kinds of blood doping and the detection features.

Direct Detection of Blood Doping

RBC Transfusion
Flow cytometry has been applied for the detection of allogeneic RBCs for over a decade. The method was first evaluated in a single-blind study on 140 blood samples (17). Most samples containing a 1.5% minor RBC population could be identified, yielding 78% sensitivity of the method. No false positive results were obtained, indicating 100% specificity (17). Recently, however, suspicion has been expressed that cheating athletes may pair up with persons with the same blood group factors thereby preventing the detection of RBC transfusion (26). The possibility exists that cheaters choose donors that suit with regard to blood group and RhD factor as well as the set of their minor antigens.

This would explain the fact that no adverse analytical findings have been reported since 2008.

Of note, there is no accredited method for the detection of re-transfused autologous RBCs, despite intensive research (52). Recently, metabolites of the plasticizer di-2-ethylhexyl phthalate (DEHP) have been proposed as markers of RBC transfusion. Autologous transfusion with RBCs stored in plastic bags causes an acute increase in urinary DEHP metabolites. The window of its detection is approximately 2 days (39).

Peptidic ESAs
Similar to the endogenous hormone, rhEpo stimulates the growth of erythrocytic progenitors in the bone marrow (Fig. 1). RhEpo can be demonstrated by chemical tests, because there are differences in the glycans of endogenous human Epo and the common rhEpo preparations (epoetins; produced in EPO cDNA-transfected mammalian cell cultures). Epo isoforms can be separated by isoelectric focusing (IEF) and detected by immunoblotting of urine samples (for an overview, see (47)). The WADA has established criteria to ensure harmonization in the performance of the tests (58). Endogenous Epo presents with more acidic isoforms than the epoetins. However, many follow-on epoetins have been developed globally (23), and their glycosylation patterns differ from those of the first copies. A detection difficulty came up with the addition of proteases (e.g. laundry detergent) to the urinary samples, as this destroys the proteins to be detected (30, 55). However, tampering of doping control samples is prohibited, including urine adulteration by proteases (58). Another issue relates to the fact that once the Hb concentration [Hb] has been raised by blood doping, only very low ESA doses are needed to maintain the elevated [Hb]. In this situation the window of rhEpo detection by IEF and immunoblotting is only 12-18h (2). The more sensitive membrane assisted isoform immunoassay (MAIIA) prolongs the window of rhEpo detection (43), but this assay is not used in all anti-doping control laboratories.

**Figure 1** Feedback-circuit of erythropoiesis and its pharmacologic manipulation. Physiologically, erythropoietin (Epo) production increases in response to hypoxia. Pharmacologically, a similar response can be evoked by stabilizing the hypoxia-inducible transcription factors (HIFs) or inhibiting GATA. Epo binds to Epo-receptors of erythrocytic progenitors and stimulates their proliferation. This action can be mimicked by distinct peptides.
Blood Doping

The mulein darbepeotin alfa is not a smart doping substance, because it has a 3- to 4-fold longer half-life (24–26 h) in circulation than rhEpo (6-8 h), and the window of its detection is prolonged to about 7 days (29, 42). Methoxy polyethylene glycol-epoetin beta (Peg-Epo) is also inept for doping as it has an ever longer half-life (6 days). IEF of Peg-Epo yields bands in the less acidic area when compared to native Epo (47). IEF for Peg-Epo detection is also applicable to blood samples (32).

Several Epo-mimetic peptides (EMPs) have been explored for treatment of anemic patients (23). EMPs are synthetic cyclic peptides of about 20 amino acids which stimulate erythropoiesis similar to Epo (Fig. 1). The seminal agent peginesatide (Omontys, originally named HematideTM, Affymax/Takeda) has been taken off market due to lethal adverse drug effects (ADEs). However, in view of recent findings indicating that the ADEs were not caused by the drug substance but by the drug product (formulated in multi-use vials), the possibility must be considered that cheating athletes may apply peginesatide in appropriate formulation (22). In addition, other EMPs such as CNTO 528 and CNTO 530 (Centocor), which have remained in the pre-approval state of clinical use, may get a second wind for therapy including misuse in sports. Therefore, it is very important to proceed in developing electrophoretic, immunological and mass spectroscopic methods for the detection of peginesatide and other EMPs in human urine and blood samples (33, 38, 57).

Drugs Activating the Endogenous Epo Gene (EPO)

Epo production is stimulated by hypoxia-inducible factors (HIFs), which form heterodimers of α- and β-subunits that activate EPO transcription (Fig. 2). The HIF-α subunits present with isoforms. The main activator of EPO, HIF-2, is composed of HIF-1β and HIF-2α (25). Acetylation and de-acetylation of HIF-2α are required for efficient HIF-2 signaling. The injection of acetate was shown to increase hematocrit (Hct) in mice (59), but the doping relevance of this effect is unknown. Under normoxic conditions, two prolyl residues of HIF-α are hydroxylated by specific prolyl hydroxylase domain proteins (PHDs). Prolyl hydroxylation results in the immediate proteolytic degradation of HIF-α. In normoxia, HIF-α can furthermore undergo asparaginyl hydroxylation by means of “factor inhibiting HIF-1α” (FIH-1), resulting in the loss of interaction with p300, a histone acetyl transferase which assists in the transcription of HIF-dependent genes. HIF-α hydroxylation can be prevented by the oral intake of certain metal ions (Fig. 2). One of these is Co²⁺, longly known in medicine to stimulate Epo production (15). Co²⁺ prevents HIF-α prolyl hydroxylation, even under normoxic conditions. Cobalt chloride was used as an anti-anemic therapeutic (daily oral doses about 100 mg) until more specific ESAs became available (15). The toxicokinetics of cobalt following oral dosing have been reviewed recently (56). Still, cobalt salt may be misused in sports, as it is readily purchasable, inexpensive and very potent. Cobalt salt doping is prohibited (58). Cobalt concentrations can be measured in urine by inductively coupled plasma-mass spectrometry (27). Note that the function of ionic Co²⁺ in stimulating EPO expression is completely separate from the role of cobalt in cobalamin (vitamin B₁₂, contains cobalt-corrin complexes) (21). Cobalamin plays a vital role in DNA synthesis and cell proliferation. The intake of cobalamin is not prohibited in sports (21).

In addition, α-ketoglutarate competitors prevent the degradation of HIF-α and stimulate the expression of EPO, because the HIF-α PHDs require α-ketoglutarate for action. Pharmaceutical companies have hand on a large number of organic chemicals (“HIF-stabilizers”) that can inhibit PHDs to increase Epo levels and Hct (46). In a Phase I trial compound FG-
Blood doping produces characteristic changes of specific RBC parameters (Table 2).

Since 2009 it is possible to sanction athletes based on indirect indicators for doping instead of proven prohibited substances.

### Hematological Parameters Associated with RBC Transfusion

Reliable detection tests are still needed to reduce the illicit use of autologous RBC transfusion. Clinical studies have shown that the blood Hb concentration ([Hb]) decreases by about 1.3 g/dL after donation of one unit of blood (~500 mL) in healthy subjects (24). Oral iron administration accelerates the recovery of [Hb], which is reached only after about 15 weeks (wks) following blood donation (24). After donation of one unit of blood, plasma ferritin levels decrease by about 30 ng/mL over a 30-day period. However, neither iron nor ferritin levels are suitable markers in anti-doping controls. Damsgaard et al. (14) subjected healthy men to withdrawal of 20% of their blood volume and replaced this by hydroxyl-ethyl starch. As a result, [Hb] was reduced by 15% for 2 wks. Due to the stimulation of Epo production, the number of reticulocytes (Ret#) was 2.4-fold increased after 7 days, remaining elevated for another wk. When 0.8 L of packed RBCs was re-infused one month later, [Hb] increased by 8%. Ret# was reduced by about 30% from day 7 to day 21 after re-transfusion (14). Ret# are known to level down to very low numbers on RBC re-transfusion (41).

### Hematological Parameters Associated with ESA Doping

Casoni et al. (13) first reported that the concentration of RBCs, [Hb], Hct, hypochromic macrerycocyte counts and the percentage of Ret (Ret%) increased, when athletes received rhEpo subcutaneously (SC) at doses of 30 units (U) per kg body weight (b.w.) every other day for 30 to 45 days, along with twice weekly intravenous (IV) iron (62 mg) and oral vitamins. The treatment with ESAs appears to increase Ret% and Ret# in two ways: (i) by increased Ret release from the bone marrow (3, 45), and (ii) by prolonged maturation of circulating Ret (28). At least following bolus injections of rhEpo a shift occurs in the circulating reticulocytes age distribution to younger cells (36). Accelerated erythropoiesis due to the use of ESAs leads to the production of iron-deficient reticulocytes (reduced mean corpuscular Hb_{mean} of Ret, MCHr). An increase in hypochromic RBCs is typically seen on rhEpo treatment despite the use of parenteral iron (11). IV iron increases the response to ESAs, and this combination is likely used by cheating athletes. Ret% usually level down to very low numbers on cessation of ESA use (3, 45).

### Procedures

WADA’s hematological “Athlete Biological Passport (ABP) Operating Guidelines” for the evaluation of RBC parameters came into force in December 2009 (58). Since then, the ABP Operating Guidelines have been continuously refined and the ABP approach has been applied by many International Federations and National Anti-Doping Organizations (ADOs). The ABP Operating Guidelines include annexes which compile mandatory protocols that must be followed by the ADOs with respect to the collection, transportation, analysis and management of the samples. This provision is necessary to ensure consistency in application, the sharing of information and the standardization of procedures.

The hematological ABP module comprises the following markers: Hct, [Hb], RBC count, Ret%, Ret#, mean corpuscular volume (MCV), mean corpuscular Hb mass (MCH), mean...
Blood Doping

and the multiparametric ABPS (Abnormal Blood Profile Score, all of the novel recombinant ESAs may be clearly recognizable, ver, doping with autologous RBCs is not directly detectable, not distance in biological fluids (“Adverse analytical finding”). Howe -

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Experiences with the ABP

According to the ABP Operating Guidelines profiles in which
the Adaptive Model identifies the [Hb] or OFF-hr score abnor-
mal with a 99.9% probability or more shall be reviewed by a
panel of three experts (58). This review shall be done anonym-
ously and come to the unanimous opinion that a prohibited
substance or method was applied. Only thereafter, ADOs proceed with the case as an asserted anti-doping rule
violation. The ABP Operating Guidelines have strengthened
the athletes’ rights. Still a matter of debate has remained with
an innocent athlete’s burden to prove the existence of a blood
anomaly as the reason for an unusual blood profile. In other
words, the athlete has to provide evidence that she or he did
not engage in doping, which is a shift in the burden of proof.
On the other side, experimental evidence exists that the sensitivity
(rate of detection of correct positives) of the hematological ABP
is insufficient (1, 10, 40). Furthermore, it has been noted that
the statistical evaluation of the data is not reliable (16). Banfi
(4) has pointed out that the statistical analysis (which is not
open to the public) is not compatible with the classical deci-
sion-making approach of medicine and science. In contrast,
the developers of the indirect persecution have praised their
approach (54). The ABP program has been introduced by sever-
ral sports associations (61). The possibility of being sanctioned
based on an abnormal ABP has likely led to a reduction in the
frequency in RBC transfusions and ESA dosages in professional
athletes.

Conflict of Interest

The author has no conflict of interest.

Table 2

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Blood Doping

The various erythropoietic agents (copies of Epo, Epo-mimetics, etc.) pose special detection diffi-
culties, when they are used at low-dose and in combination. De-
tection methods for the various chemical drugs (HIF-stabilizers
and GATA-inhibitors) that increase Epo production and ery-
thropoiesis have been developed but may still not suffice. EPO
transfer is imaginable, yet it is medically not well-engineered.

To overcome deficiencies in the direct detection of blood
doping, the ABP has been introduced, which is based on the
monitoring of selected RBC parameters. Blood doping is sus-
pected, when these parameters change in a non-physiological
way. The hematological ABP approach takes into account the
physiological variations due to training and competitions (49),
and to hypoxia-exposure situations (50, 51).

Evaluators must come to the unanimous opinion that a
prohibited substance or method was applied. Only thereafter,
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Indicators of blood transfusion or ESA doping. ↑ Increase; ↓ Decrease; ↔ No change, according to Sottas et al. (54).

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Corpuscular Hb concentration (MCHC), RDW-SD (red cell
distribution width [standard deviation]) and IRF (immature
reticulocyte fraction) (58). Additional parameters can be the
mean Ret cell volume (MCVr), the mean Ret Hb concentration
(MCHCr) and the mean Ret Hb mass (MCHr). Calculated param-
eters are the OFF-hr score (index of stimulation derived from
the formula: [Hb] (g L⁻¹) - 60x √ Ret%; normal range: 85-95) (18)
and the multiparametric ABPS (Abnormal Blood Profile Score,
which considers Hct, [Hb], RBC count, Ret%, MCV, MCH and
MCHC (53). The Bayesian Inference Model (“Adaptive Mod-
el”) for evaluation incorporates individual longitudinal RBC
parameters and factors for heterogeneous populations (48). It
is used adaptively to predict the likely profiles for future sam-
pies. Thereby, a certain percentage of false positives is accep-
ted (Article 3.1 of the WADA Code: “This standard of proof in all
cases is greater than a mere balance of probability but less than
proof beyond a reasonable doubt.”). Only [Hb] and OFF-hr score
presently fulfill the requirements to sanction an athlete. [Hb]
shows normally little intra-individual variation (coefficient of
variation <5%) (37). Therefore, larger deviations are suspicious
for doping. On cessation of effective ESA treatment the OFF-
hr score increases (18). The other ABP markers can be used as
additional evidence to distinguish between blood doping, al-
tered quality of the blood sample (e.g. hemolysis) and/or the
identification of a possible pathological condition. Zorzoli (60)
has provided vivid illustrations of typical normal and abnormal
hematological ABP profiles.

According to the ABP Operating Guidelines profiles in which
the Adaptive Model identifies the [Hb] or OFF-hr score abnor-
mal with a 99.9% probability or more shall be reviewed by a
panel of three experts (58). This review shall be done anonym-
ously and come to the unanimous opinion that a prohibited
substance or method was applied. The reviewers are expected
to be able to analyze and certify whether a blood value abnor-
mality is the result of doping, or due to an acute disorder re-
spectively a genetic variation. Here, explanations given by the
athlete must also be considered, for example information on
recent exposure to high altitude or extreme heat conditions.

Experiences with the ABP

Mørkeberg et al. (40) re-transfused 29 subjects with either one or
two units of autologous blood in a comparative study of three
blood passport approaches and four blood markers. One of the
main conclusions of the study was that both the sensitivity (rate
of detection of correct positives) and the specificity (lack of false
positives) varied greatly among the statistical methods (40).
When Ashenden et al. (1) treated ten subjects twice weekly with
low-dosed rhEpo IV for up to 12 wks, Hb↑ increased by 10%. Still,
the ABP software (specificity set at 99.9%) did not flag any
subjects as being suspicious of doping whilst they were receiv-
ing rhEpo (1). Berno et al. (10) treated 24 subjects with rhEpo
(three different drug regimens) and then evaluated the ABP pa-
rameters: [Hb], Ret% and OFF-hr score. This screening indicated
rhEpo treatment only in 58% of the subjects (10). In a single case
report, the ABP failed to flag the use of the HIF-stabilizer FG-
4592, which was eventually discovered by chemical analysis in
the urine of the athlete (8).
Positive and negative regulation of Second-generation blood tests to detect erythropoietin abuse by doping strategies. Haematologica. 2006; 91: 1006-1008.


Blood Doping


(48) Robinson N, Sottas PE, Mängin P, Sauvy M. Bayesian detection of abnormal hematological values to introduce a no-start rule for heterogeneous populations of athletes. Haematologica. 2007; 92: 1143-1144. doi:10.3324/haematol.111682


