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Features of Blood Doping

Merkmale von Blutdoping

REVIEW

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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 O_2 to muscles and therefore increase performance.

› **Artificial O_2 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 O_2-labile α-subunits can be prevented by the oral intake of cobaltous (Co^{2+}) 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 widespread in sports.

› **The World Anti-Doping Agency (WADA)** has implemented 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.

Zusammenfassung

› **Blutdoping** beschreibt den Gebrauch bestimmter Techniken und/oder Substanzen, um die Gesamtmasse der roten Blutzellen zu erhöhen, sodass mehr O_2 transportiert und da-mit die körperliche Leistung verbessert wird.

› **Künstliche O_2-Träger** spielen im Sport offen-bar keine Rolle. Infundiertes Fremdblut ist – wg. unterschiedlicher Blutgruppenmerkmale – nachweisbar, re-transfundiertes Eigenblut dagegen nicht. Rekombinantes humanes Erythropoietin (rhEpo) und seine Analoga sind mittels isoelektrischer Fokussierung und Immunoblotting detektierbar. Es gibt peptidische Epo-Mimetika, aber keines dieser Produkte ist klinisch zugelassen. Die Expression des Epo-Gens (EPO) wird durch hypoxie-induzierbare Transkriptionsfaktoren (HIF) stimuliert, welche aus einer α- und einer β-Untereinheit bestehen.

› **Die Hydroxylierung** der O_2-labilen α-Untereinheit lässt sich durch die Einnahme von Kobalt(II)-Salzen oder α-Ketoglutarat-Kompetitoren verhindern. Diese – strukturell simplen – Stoffe sind oral wirksam (sog. „HIF-Stabilisatoren"). Denkbar ist außerdem der missbräuchliche Einsatz von Inhibitoren der GATA-Genregulatorproteine, wodurch der EPO-Promotor aktiviert wird. EPO-Gentransfer ist dagegen wohl im Sport (noch) nicht verbreitet.

› **Die WADA** hat 2009 Richtlinien für die indirekte Suche nach Blutdoping herausgegeben ("Athlete Biological Passport (ABP) Operating Guidelines"). Dabei werden individuell longitudinal verschiedene Erythrozyten-Parameter bewertet (u.a. Hämoglobinkonzentration [Hb], Hämatokrit, Erythrozytenzahl, Retikulozyten-Zahlen [Ret], mittleres Erythrozytenvolumen [MCV] und mittlere Hb-Masse der einzelnen Erythrozyten [MCH]). Sportrechtlich relevant sind primär [Hb] und OFF-hr score ([Hb] - 60√ Ret %; Normalbereich: 85-95). Die Validität des ABP-Verfahrens ist Gegenstand aktueller Forschung.

Introduction

The total mass of hemoglobin (Hb_{mass}) correlates with the rate of maximal O_2 uptake (VO_{2max}) (reviewed in (23)). Red blood cell (RBC) transfusion maneuvers increase VO_{2max} 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 O_2 in lungs and tissues, increased percentage of young red cells with good functional properties (in response to ESA treatment), increased buffer capacity, increased blood volume, vasoconstriction, reduced damage by radicals, and mood improvement by cerebral effects of ESA. ›

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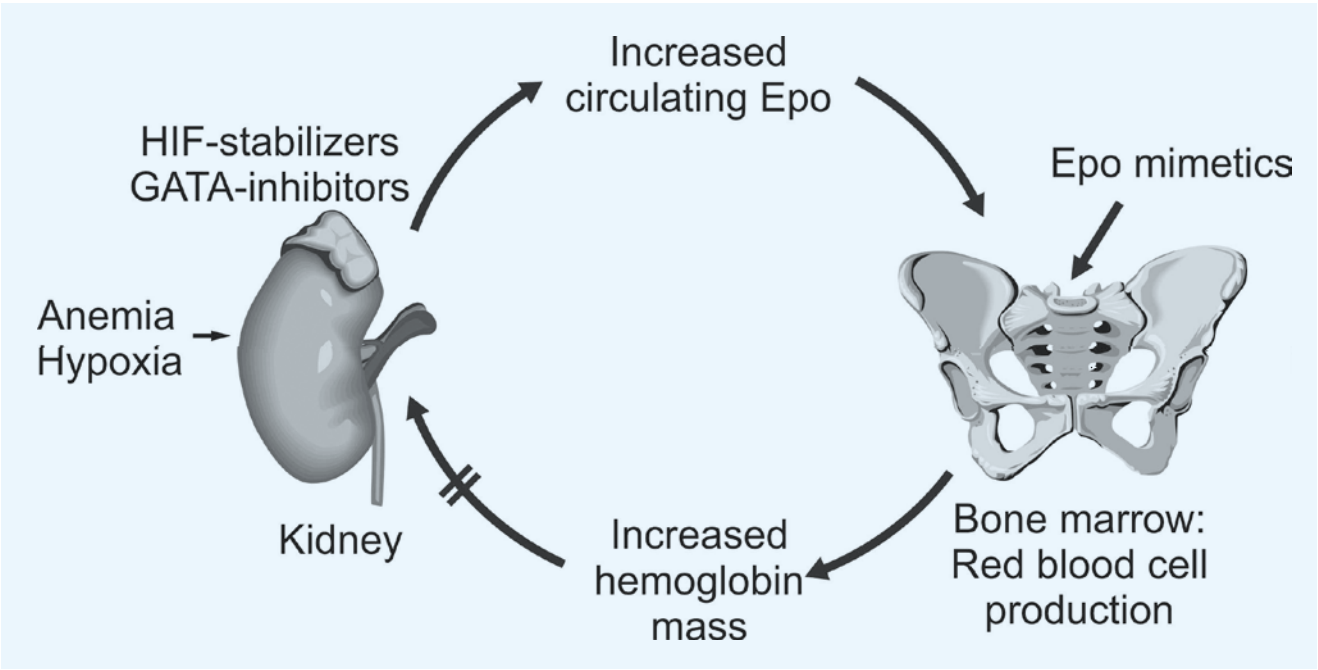

Figure 1

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.

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 O_2 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 O_2 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.

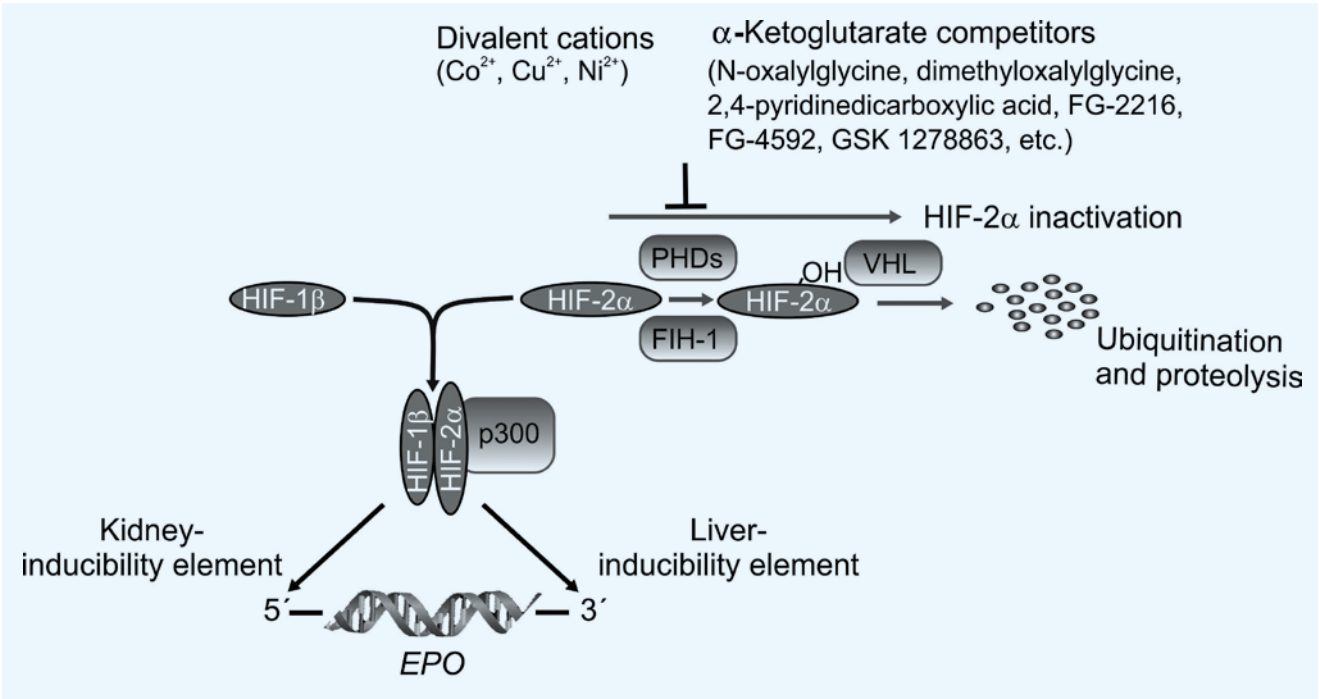

Figure 2

The hypoxia-inducible transcription factors (HIFs) increase erythropoietin gene (EPO) expression in the kidneys and the liver. However, under normoxic conditions the HIF-α subunits (most important HIF-2α) are prolyl hydroxylated by prolyl hydroxylase domain proteins (PHDs; most important PHD2). In turn, the von-Hippel Lindau protein (pVHL)-E3-ubiquitin ligase complex binds to HIF-α, which then undergoes proteasomal degradation. This process can be prevented by chemicals such as cobaltous (Co^{2+}) salt or α-ketoglutarate competitors („HIF-stabilizers"). HIF-α can also be asparaginyl hydroxylated by the enzyme "Factor-Inhibiting HIF-1" (FIH-1), which results in loss of binding of the transcriptional co-activator p300.

The mutein darbepoetin 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 Co^{2+}, longly known in medicine to stimulate Epo production (15). Co^{2+} 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 Co^{2+} in stimulating EPO expression is completely separate from the role of cobalt in cobalamin (vitamin B_{12}, 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- ›

Table 1

Orally active HIF stabilizers (HIF prolyl hydroxylase inhibitors) presently in clinical trials. INN, International Nonproprietary Name; https://clinicaltrials.gov [December 2015].

SUBSTANCE (INN)	DRUG COMPANY	CLINICAL TRIAL PHASE (MOST ADVANCED)
FG-4592 (Roxadustat)	Fibrogen/Astella Pharma/ AstraZeneca	III
BAY85-3934 (Molidustat)	Bayer	II
GSK1278863 (Daprodustat)	GlaxoSmithKline	II
AKB-6548	Akebia Therapeutics	II
JTZ-951	Akros pharma	I
DS-1093a	Daiichi Sankyo	I

2216 (Fibrogen) proved to increase the level of circulating Epo not only in nephric but also in anephric patients (7). This finding has demonstrated that HIF-stabilizers stimulate Epo production at extrarenal sites such as the liver, too. At least six PHD inhibitors are presently tested in clinical trials (Table 1). Pharmacologists have considered the therapeutic potential of HIF-stabilizers also in clinical states of tissue hypoxia and injury. A two-week study of GSK 1278863 (GlaxoSmithKline) in patients with claudication at a dose below that necessary to increase [Hb] found no improvement of ischemic symptoms, but indicated a decrease in total cholesterol, low density lipoprotein and high density lipoprotein (44). Currently available methods and strategies for the determination of selected HIF-stabilizers in sports drug testing are based on liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) (8). Recently, the first case has been reported of an athlete (a sporting walker) who used a HIF-stabilizer (FG-4592) for doping purposes (12).

Epo production is also stimulated by GATA-2 inhibitors. GATA-2 belongs to the GATA transcription factors, which contain zinc fingers in their DNA binding domain and bind to the DNA sequence "GATA" (from the nucleobases: guanine, adenine, thymine, adenine). GATA inhibitors are non-peptidic organic compounds that prevent GATA-2 from suppress-ing the EPO promoter (19). F.e., the diazepane derivative K-7174 acts this way. Its follow-on product K-11706 exerts even stronger erythropoietic effects, and it has proved to increase physical performance in mice (20). The chemical structure of K-11706 is undisclosed. Latterly, studies with K-11706 or other erythropoiesis-stimulating GATA inhibitors have not been reported.

EPO Transfer

Autologous ex vivo EPO transfer has been explored for clinical purposes. Thereby, dermal core samples are re-transplanted following transfection with EPO complementary DNA (cDNA) (34). However, the method has not stepped beyond the clinical trial phase for ten years. In vivo EPO transfer would probably be detectable. Unusual Epo glycosylation forms were apparent on allogeneic EPO transfer into skeletal muscle of cynomolgus macaques (31). Tests have been developed to detect transgenic DNA in blood (5, 6), making use of the fact that cDNA does not contain introns. Taken together, EPO gene doping is unlikely to be applied in sports, at present.

Indirect Signs of Blood Doping

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 macrocyte 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_{mass} 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).

WADA's Biological Passport

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

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 parameters 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 Model") for evaluation incorporates individual longitudinal RBC parameters and factors for heterogeneous populations (48). It is used adaptively to predict the likely profiles for future samples. Thereby, a certain percentage of false positives is accepted (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, altered 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 abnormal with a 99.9% probability or more shall be reviewed by a panel of three experts (58). This review shall be done anonymously 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 abnormality is the result of doping, or due to an acute disorder respectively 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 three 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_{mass} 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 receiving rhEpo (1). Børno et al. (10) treated 24 subjects with rhEpo (three different drug regimens) and then evaluated the ABP parameters: [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).

Comments

Traditional anti-doping analyses aim at demonstrating a substance in biological fluids ("Adverse analytical finding"). However, doping with autologous RBCs is not directly detectable, not all of the novel recombinant ESAs may be clearly recognizable, the time-frame for their detection is limited, and there may be urine manipulation. The numerous new erythropoietic agents (copies of Epo, Epo-mimetics, etc.) pose special detection difficulties, when they are used at low-dose and in combination. Detection methods for the various chemical drugs (HIF-stabilizers and GATA-inhibitors) that increase Epo production and erythropoiesis 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 suspected, 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, 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 decision-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 several 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.

Table 2

Indicators of blood transfusion or ESA doping. ↑ Increase; ↓ Decrease; ↔ No change, according to Sottas et al. (54).

INDICATOR	BLOOD TRANSFUSION		ESA DOPING	
	WITHDRAWAL	RE-INFUSION	STIMULATION	MAINTENANCE
Hemoglobin Concentration	↓	↑	↑	↑
Hematocrit	↓	↑	↑	↑
Red Blood Cell Counts	↓	↑	↑	↑
Reticulocytes (%)	↑	↓	↑	↓
OFF-hr Score	↓	↑	↔	↑

Conflict of Interest

The author has no conflict of interest.

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