BIOCHEMISTRY, PHYSIOLOGY, AND COMPLICATIONS OF BLOOD DOPING: Facts and Speculation

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BIOCHEMISTRY, PHYSIOLOGY, AND COMPLICATIONS OF BLOOD DOPING: Facts and Speculation

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□ *Competition is a natural part of human nature. Techniques and substances employed to enhance athletic performance and to achieve unfair success in sport have a long history, and there has been little knowledge or acceptance of potential harmful effects. Among doping practices, blood doping has become an integral part of endurance sport disciplines over the past decade. The definition of blood doping includes methods or substances administered for non-medical reasons to healthy athletes for improving aerobic performance. It includes all means aimed at producing an increased or more efficient mechanism of oxygen transport and delivery to peripheral tissues and muscles. The aim of this review is to discuss the biochemistry, physiology, and complications of blood doping and to provide an update on current antidoping policies.*

Keywords Antidoping testing, blood doping, doping, erythropoietin, sport medicine.

Abbreviations **ADP**, adenosine diphosphate; **ATP**, adenosine triphosphate; **CERA**, continuous erythropoiesis receptor activator; **Epo**, erythropoietin; **HBOC**, hemoglobin-based oxygen carrier; **HIF**, hypoxia inducible factor; **PFC**, perfluorocarbon emulsion; **pO_2**, oxygen partial pressure; **RBC**, red blood cell; **rHuEpo**, recombinant human erythropoietin; **RSR**, 2-[4-[(3,5-dichlorophenylcarbamoyl)-]methyl]-phenoxy]-2-methylpropionic acid; **sTfr**, soluble transferrin receptor.

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I. INTRODUCTION

Competition, a natural part of human nature, has been crucial from both evolutionary and survival perspectives.[1] The ability to optimize muscular power output is considered fundamental to the successful performance of many athletic and sporting activities, and peak performance has been traditionally achieved by regular training, sophisticated techniques, and good overall health and fitness.[2] Although success in competition is traditionally achieved through one or more of the previously mentioned scenarios, some athletes seek to take a step further. Since ancient Greco-Roman times, fame, celebrity, and economic benefit arising from success in fighting or competition have persuaded some athletes to use artificial, and often unfair and dangerous, means to enhance their athletic performance.[3] According to a common position shared by most international governing bodies of sport, any sporting practice should be banned when it causes injury or it gives an athlete an unfair technological or athletic advantage that is too expensive or greatly innovative for most other competitors.[3] Outstanding advances in basic and applied biochemistry have contributed enormously to the development of increasingly sophisticated and complex performance-enhancing substances and techniques. Among such techniques, blood doping has regrettably become an integral part of sport and fair play. The term "blood doping" or "blood boosting," earlier known as "induced erythrocythemia," usually refers to methods or substances administered for non-medical reasons to healthy athletes with the aim of increasing maximal aerobic power and thereby improving aerobic performance (Table 1).[4] In this review, the context in which blood doping is abused will be discussed.

II. REVIEW OF MUSCULAR ENERGETICS

A comprehensive review on muscular metabolism and energy expenditure has recently been published by Rose and Richter.[5] From the energy-producing standpoint, the most important molecule in biology is adenosine triphosphate (ATP). The time course of energy metabolism during moderate exercise involves primarily the phosphagen system (for the first 10 to 15 s), followed by anaerobic glycolysis for the next 1 to 2 min and aerobic metabolism for physical activities lasting more than 2 min. ATP molecules normally present within muscle cells can be promptly used to sustain muscle contraction; phosphocreatine is an additional reserve of energy that can be used to rapidly synthesize ATP. Both systems provide energy at a very rapid rate, but when a muscle fiber is undergoing a sustained contraction, these energy reserves are quickly exhausted. When muscle fibers are actively contracting, each thick filament breaks down roughly 2500 ATP molecules/s.

Because even a small skeletal muscle contains thousands of muscle fibers, the ATP demands are enormous and, therefore, skeletal muscles must rely on

TABLE 1 Blood Doping Techniques

a)	Blood transfusion
	1) Autologous blood transfusion
	2) Allogeneic blood transfusion
b)	Erythropoiesis-stimulating substances
	1) Recombinant human erythropoietin (rHuEpo)
	i) Epoetin alfa (Epogen®, Eprex®, Epoxitin®, Epypo®, Erypo®, Espo®, Globuren®, Procrit®)
	ii) Epoetin beta (Epogin®, Marogen®, NeoRecormon®, Recormon®)
	iii) Epoetin gamma
	iv) Epoetin delta (Dynepo™)
	2) Darbepoetin alfa, a novel erythropoiesis-stimulating protein or NESP (Aranesp®)
	3) Continuous Erythropoiesis Receptor Activator or CERA
c)	Hypoxic training
	1) Artificial altitude environments or facilities
	2) Hypoxic gas mixtures
	3) Supplemental oxygen breathing
d)	Blood substitutes
	1) Perfluorocarbon emulsions
	2) Hemoglobin-based oxygen carriers
	3) Allosteric modulators of hemoglobin
e)	Supplementation therapies
	1) Iron
	2) Cobalt chloride
f)	Gene doping
	1) Human erythropoietin gene transfection
	2) Regulation of the HIF pathway

alternative mechanisms to supply energy. Most cells generate ATP through aerobic metabolism in mitochondria and glycolysis in the cytoplasm. Aerobic metabolism normally provides up to 95% of the energy demand of a resting cell. In this process, mitochondria absorb oxygen, adenosine diphosphate (ADP), phosphate ions, and organic substrates that enter the tricarboxylic acid cycle (also known as the citric acid cycle or the Krebs cycle). While carbon atoms are released as carbon dioxide, hydrogen is shuttled to respiratory enzymes in the inner mitochondrial membrane where their electrons are removed. After a series of intermediate steps, protons and electrons are combined with oxygen to form water. By this process, a large amount of energy is efficiently produced, as each organic molecule fed to the tricarboxylic acid cycle generates 17 ATP molecules.

During extensive physical exercise, the demand for energy, along with mitochondrial ATP production, progressively increase to a maximum rate that is determined by the availability of oxygen, which cannot diffuse into the muscle fiber fast enough to enable the mitochondria to fulfill the ongoing energy expenditure. At peak levels of exertion, mitochondrial activity can provide only about one-third of the ATP required. Therefore, oxygen becomes progressively depleted, and muscles cannot get sufficient amounts to perform at their optimal potential, in terms of both power and resistance.

Such a relative gap between oxygen demand and availability is conventionally called "oxygen debt."[6]

Owing to the relative depletion of oxygen to produce ATP via traditional mechanisms, muscle tissue is compelled to shift to the anaerobic pathway, culminating in ATP production through conversion of pyruvic acid, provided by the enzymatic pathway of glycolysis, to lactic acid. Therefore, the process of anaerobic glycolysis enables the generation of additional energy when mitochondria are unable to fulfill the current energy demand. However, anaerobic energy production has its drawbacks. Although nearby 80% of the lactate produced diffuses from the muscles and is transported to the liver for conversion to glucose or glycogen, in conditions of extensive training, it cannot be completely cleared and lactate gradually accumulates at both the site of synthesis and in the blood.

As the relative concentration of intracellular lactate can become extremely elevated in muscles, this process can persist for several minutes after the end of the exercise. As the progressive accumulation of lactate lowers the intracellular pH and alters the functional characteristics of key enzymes, the overall efficiency of the muscular contraction finally declines, producing the characteristic symptoms of fatigue, pain, and muscle soreness that may develop several hours or even days after particularly strenuous or unaccustomed exercise. Lactic acidosis typically occurs when the concentration of lactate in blood exceeds 4 mmol/l. Highly trained athletes have maximal oxygen uptakes that may be double those of sedentary people and that permits greater muscular activity coupled to a reduced production and accumulation of lactic acid.

Because of this physiological drawback, it becomes clear that the limiting steps to effective energy production in muscles during demanding physical exercises are: (a) the availability of intracellular energy substrates (glucose) and reserves (fat, glycogen, proteins); (b) an efficient circulatory supply; and (c) sufficient blood oxygenation. Anything that interferes with any of these factors promotes premature muscle fatigue and compromises performance.[7] Oxygen is carried to peripheral tissues and muscles by two efficient delivery systems: 3% is carried in solution (plasma), whereas the remaining 97% is bound to hemoglobin, the main protein in red blood cells. Practices that are aimed at producing an increase in hemoglobin in blood or a more efficient mechanism of oxygen transport and delivery are associated with improved energy production, allowing the muscles to become more fatigue resistant and to perform better.

III. TRANSFUSION

In 1628 the English physician William Harvey described the blood circulatory system. Shortly afterward, the first reported blood transfusion was attempted.[8] Since than, substantial improvements have been made in the

techniques employed for blood transfusion. Blood transfusions were originally used to support critically ill patients with severe forms of acute and chronic anemia. However, recent advances in biotechnology have allowed the separation of whole blood in its components. Because patients seldom require all of the components of whole blood, it makes sense to transfuse only that portion needed for a specific condition or disease. This treatment is conventionally referred to as "blood component therapy."[9] Typically, up to four components may be derived from 1 unit of blood: red blood cells (RBCs), platelets, plasma, and cryoprecipitated antihemophilic factor (AHF).[10] RBCs may be stored under refrigeration for a maximum of 42 days, or they may be frozen for up to 10 years. Platelets must be stored at room temperature and may be kept for a maximum of five days. Fresh frozen plasma, mainly used for the therapy of acquired and congenital bleeding disorders, is stored frozen for usually up to one year. Cryoprecipitated AHF, which contains one or more specific clotting factors, is made from fresh frozen plasma and may be stored frozen for up to 1 year.[11] Granulocytes, separated from whole blood and occasionally used to fight infections, must be transfused within 24 h of donation.[12] Additional products manufactured from whole blood include albumin, immune globulin, specific immune globulins, and clotting factor concentrates. Commercial manufacturers commonly produce these blood products.[13]

Blood transfusions can be traditionally classified as autologous, where the blood donor and transfusion recipient are the same, or as allogeneic, where the blood is transfused into someone other than the donor. The most common autologous donation is the preoperative donation of blood for possible re-transfusion up to six weeks before or following elective surgery.[14] Potential autologous blood donors are medically stable patients free of infection. As a significant amount of iron is removed by each autologous donation, an adequate time for recovery of not less than 72 h from the last donation, and appropriate iron supplements, are usually required for patients undergoing autologous donations. Nearly 50% of autologous donations are not used by the donor and are discarded, as current standards do not allow transfusion of these units to another patient for safety reasons.

An important step in ensuring the safety of allogenic transfusions is the screening of donated blood for infectious diseases. Today, nine tests for infectious diseases are traditionally performed.[15] Hepatitis B (HBV) and syphilis tests were in place before 1985. Since then, tests for human immunodeficiency virus (HIV-1 and HIV-2), human T-lymphotropic virus (HTLV-I and -II), and the hepatitis C virus (HCV) have been introduced. There have been occasional reports of highly probable transfusion-associated iatrogenic variant Creutzfeldt-Jakob disease infection, which is responsible for a rare degenerative and fatal nervous system disorder.[16] During the last decade, significant progress has been made in improving both the sensitivity and specificity of tests using brain and lymphoreticular tissues to identify Creutzfeldt-Jakob

disease-infected individuals. However, no sensitive, specific, and reliable diagnostic screening test for early identification of infected individuals that would ensure the safety of the blood supply has been developed to date.[17]

Current evidence suggests that blood transfusions are unlikely to be beneficial in the absence of active blood loss when the hemoglobin concentration exceeds 100 g/l (hematocrit >30%). The benefits arising from blood transfusions may exceed the risks when the hemoglobin concentration falls to 70 g/l (hematocrit <21%).[18] Therefore, the majority of existing guidelines conclude that transfusion is rarely indicated when the hemoglobin concentration is greater than 100 g/l, and it is almost always indicated when it falls below a threshold of 60 g/l in healthy, stable patients or is likely a higher threshold in older or sicker patients. In particular clinical circumstances, such as anesthetized patients, this threshold should be modulated by factors related to the dynamic nature of surgery.[19]

It was observed earlier that, in endurance sports such as cycling, triathlon, cross-country skiing, and marathon running, ways of boosting the blood's oxygen-carrying capacity can enhance performance by over 20%. The first clear evidence of blood doping through blood transfusion came from a controlled experiment carried out in 1947.[20] Since then, transfusions have long been used for this purpose, as they are an extremely straightforward, simple, and effective method of increasing the oxygen carrying capacity of blood.[21] However, transfusions carry significant risks, such as the contraction of infectious diseases and life-threatening immune reactions (allergic reactions, acute and delayed immune hemolytic reactions, graft-versus-host disease). Following the commercial availability in the early 1990s of natural or recombinant human erythropoietin stimulating substances (rHuEpo), a black market for it quickly developed. It was not until some years later that a reliable urine-based test for detecting rHuEpo became available. Then old-fashioned blood transfusions made a strong comeback, despite the health risks, as recently testified by the presumptive non-negative case of the American cyclist Tyler Hamilton (Hamilton fails pair of drug tests—American cyclist could lose gold. *Washington Post*, September 24, 2004:D01).

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There are two methods of doping through blood transfusions: autologous and allogeneic; for convenience and safety, the former are reportedly much more used. The traditional procedure of autologous blood transfusion begins by the withdrawal of 1 to 4 units of blood (1 unit = 450 ml of blood) several weeks before competition. The blood is centrifuged, the plasma components are immediately reinfused, and the corpuscular elements, principally RBCs, are stored refrigerated at 4°C or frozen at −80°C.[22] As blood stored by refrigeration display a steady decline in the number of RBCs, a substantial percentage, up to 40%, of the stored RBCs may not be viable.[23] The freezing process, conversely, limits the aging of the cells, allowing the storage of the blood for up to 10 years with a 10% to 15% loss of RBCs.[24] Stored RBCs are then reinfused, usually 1 to 7 days before a high-endurance event.

When properly performed, this process increases hemoglobin and hematocrit levels by up to 20%, but it is not completely safe and free from side effects. A large infusion of RBCs may be associated with hyperviscosity syndrome, which is characterized by increased blood viscosity and decreased cardiac output and blood flow velocity and results in reduction of peripheral oxygen delivery. Additional complications may be phlebitis, septicemia, bacterial infection, and air/clot embolism.[24] In addition, allogeneic transfusions may trigger transfusion reactions characterized by fever, urticaria, and anaphylactic shock and are significantly associated with transmission of blood-borne infectious diseases including hepatitis, acquired immunodeficiency syndrome, malaria, cytomegalovirus, and Creutzfeldt Jacob disease.[25] There are anecdotal reports of athletes (the 1984 United States Olympic cycling team and the Spanish cyclist Manzano among others) experiencing severe side effects from blood transfusions, and some of them nearly died after being injected with poorly stored blood (Fotheringham W. It can kill, but blood doping is in vogue again. *The Guardian*, September 24, 2004). Earlier studies have shown that a greater than 5% increase in circulating hemoglobin is necessary to improve performance, suggesting that athletes would need to infuse at least 1 unit of blood to obtain a surreptitious athletic advantage.[23]

For the last decade, the only way to test for blood doping by allogeneic transfusion has been to assess the hematocrit or hemoglobin threshold; several sport federations banned athletes if random tests gave values over an arbitrary limit.[26] Recently, a more reliable approach to the detection of blood transfusion in athletes has been proposed. The method is based on the quantification of antigenically distinct donor and recipient RBCs by flow cytometry, through the use of standard blood bank antisera in combination with a fluorescent-labeled secondary antibody directed against human immunoglobulin. This strategy allows detection of even a single unit of blood transfused, provided that there is at least one antigen mismatch between donor and recipient.[27,28] As individual RBCs display an almost unique antigen pattern, which is strict under genetic regulation, the possibility of two blood samples matched for ABO and Rh(D) being identical for the panel of 12 blood group antigens tested is less than 1:500. The test currently involves RBC phenotyping for the antigens C, c, E, e, K, k, Fya, Fyb, Jka, Jkb, S, and s.[28] Therefore, flow cytometry appears to be the ideal technique for detecting allogeneic transfusion with a high degree of analytical efficiency. However, this test has some limitations. First, it does not detect autologous transfusion with blood donated and stored beforehand, as the RBCs surface antigens of the donor and recipient are identical. An additional problem is the lack of an easy confirmatory test, such as DNA analysis. A final concern arises from persons who are blood group chimeras, as the identification of a second RBC population may indicate either a transient (allogeneic RBC transfusion) or static (life-long chimera) phenomenon. However, serial testing will enable discrimination between these two situations.[29]

Many of these innovative methods have been tested and proven efficient in other fields of medicine, for example criminology, but a definitive answer for doping analysis is yet to come. Another strategy that has been suggested recently is the use of a hematologic passport, which is based on the sequential evaluation of some hematological and biochemical parameters. An athlete's hematologic profile should be fairly stable over time.[30] Using proper sequential determinations, individual reference ranges can be defined for both hemoglobin and hematocrit. Thus far, the hematologic passport appears to be a feasible solution to identify athletes with non-physiological variations within a global strategy to deter blood doping, but it has not been approved, nor is it being incorporated into developing antidoping policies.

IV. ERYTHROPOIESIS-STIMULATING PROTEINS

The human erythropoietin gene was originally cloned in the mid 1980s,[31] and rHuEpo was first approved for marketing in France in 1988. Its therapeutic use for the treatment of several forms of chronic anemia was shown to be effective and safe.[32] On this basis, genetically engineered erythropoietin (Epo) has been systematically administered to anemic chronic renal failure patients for the past 15 years. By the mid-1990s, it had also become clear that Epo was the drug of choice among endurance athletes seeking to improve performance.[33] From a technical perspective, Epo has several advantages over blood transfusion, including no complex logistically challenging maneuvers such as blood withdrawal, storage, and reinfusion. In addition, there is no decay in performance or training after a period of blood withdrawal, and there is limited "detectability," as Epo is a naturally occurring peptide hormone.[34] The use of Epo in international competition has been highlighted by a number of scandals, and its misuse as an ergogenic aid has been estimated to range from 3 to 7% of elite endurance sport athletes.[35] A considerable amount of clinical evidence supported the use of rHuEpo for doping purposes, as it provided significant erythropoietic and ergogenic benefits due to substantial increases in hemoglobin, hematocrit, maximal oxygen uptake, and exercise endurance time.[35]

A. Biochemical and Biological Properties of Human Erythropoietin

Epo is a naturally occurring hormone. Its existence was suspected almost a century ago by Carnot and Deflandre, who postulated that a humoral factor, which they called "hemopoietine," regulates RBC production.[36] The definitive existence of Epo was demonstrated by Krumdieck in 1943.[37] The sequence encoding for human Epo is located on chromosome 7.[38] Human Epo, a 165-amino acid glycoprotein with a molecular weight of 34 kDa, is derived from a 193-amino acid transcript that has been modified by the cleavage of a

27-amino acid leader sequence and the loss of the carboxyl-terminal arginine by post-translational processing by an intracellular carboxypeptidase.[39,40] After synthesis, N-linked carbohydrate chains with terminal sialic acids prevent the immediate hepatic clearance of the molecule.[41] Despite few sequence homologies, the tertiary structure of Epo is similar to that of other growth hormones and cytokines such as growth hormone, prolactin, interleukin-6, and granulocyte colony-stimulating factor.[42]

Thus far, several mammalian Epo genes have been cloned, sequenced, and expressed. Epo is highly conserved among mammals, and there is a high degree of sequence homology in the coding region of the mature secreted protein. More than 63% of the molecule is composed of invariant amino acids (106 residues); human and monkey Epos display 94% and 91% sequence homology in nucleotides and amino acid composition, respectively. In contrast, human and mouse Epos are 76% identical in nucleotide sequence and 80% identical in amino acid sequence.[43] Temporal and tissue-specific signals limit expression of the Epo gene primarily to specific cell lines, namely, subpopulations of hepatocytes and Ito cells in fetal liver[44] and fibroblast-like type I interstitial cells in adult kidney.[45] There is also increasing evidence that many non-hematopoietic organs and tissues, such as stem cells;[46] the embryo proper, including its developing nervous system;[47] brain;[48] uterus;[49] and ovary express Epo.[50] Minimal levels of Epo expression were also detected in the lung, spleen, and testis of rats.[51]

As testing strategies for Epo are as important as the development of reliable analytical procedures, detailed knowledge of the pharmacokinetic characteristics of rHuEpo is essential both for interpreting changes observed in indirect markers of administration and for planning suitable methods of testing for direct identification. Following subcutaneous administration, a "flip-flop" phenomenon can be observed; the pharmacokinetics appear to be linear from 50 to 1000 U/kg, but not for a lower dose of 10 U/kg.[52] The mean half-life of 50 IU/kg daily repeated subcutaneous administration of rHuEpo is about 35.5 h, and the total clearance is 17 ml/h/kg. Remarkably, the total clearance appears nearly three times higher in athletes than in untrained subjects (6.5 ml/h/kg). The half-life is five times longer following subcutaneous than intravenous administration (from 4 to 7 h).[53] The administration of rHuEpo is typically accompanied by a delayed increase in hemoglobin (up to 9.6%), hematocrit (up to 8.3%), reticulocytes, macrocytes, serum Epo, and soluble transferrin receptor (sTfr) concentrations.[54,55] The increase of these markers becomes significant from the third to the tenth day following initial administration, and it is always followed by a negative and transitory feedback loop of endogenous Epo production, which can be interpreted as an indirect marker of rHuEpo administration.[52] In clinical practice, rHuEpo administration produces a remarkable acceleration of the dynamic response of maximal oxygen uptake to submaximal exercise, increasing exercise capacity.[55]

Several variants of rHuEpo are currently available. The term "epoetin" conventionally refers to rHuEpo preparations that have an amino acid sequence homologous to that of the naturally occurring hormone, whereas the appropriate Greek letters, "alfa," "beta," "gamma," and "delta" designate preparations that differ in composition and/or the nature of the carbohydrate moieties. Epoetin alfa (Epogen®, Eprex®, Epoxitin®, Epypo®, Erypo®, Espo®, Globuren®, Procrit®) and epoetin beta (Epogin®, Marogen®, NeoRecormon®, Recormon®), which are produced by transfected Chinese hamster ovary cells and are nearly biochemically and immunologically identical to their naturally occurring counterpart, display similar molecular and pharmacokinetic characteristics, although the latter has a higher molecular weight, a lower number of sialylated glycan residues, and, possibly, slight pharmacokinetic advantages, such as a longer terminal elimination half-life.[56,57]

Epoetin beta has been the subject of studies aimed at proving efficacy with a reduced administration frequency, but results are rather controversial. Epoetin gamma is produced in a different host cell than the other erythropoietic agents; therefore, its glycosylation pattern and pharmacokinetics are different. Small-scale clinical studies found epoetin gamma to be slightly more biologically active than epoetin alfa.[57] Epoetin delta (Dynepo™) is a recently approved rHuEpo produced by human cells engineered to transcribe and translate the Epo gene under the control of a newly introduced regulatory DNA sequence. Epoetin delta has the same amino acid sequence and glycosylation pattern as human Epo. Because it has become available recently, only limited clinical data are available. The pharmacodynamic response to two to three times weekly 40 to 100 IU/kg epoetin delta administration appears to be consistent with known Epo pharmacological activity and includes a dose-dependent increase in hematocrit (between 0.12 and 0.18%/day), hemoglobin, reticulocytes, and RBCs for at least 3 to 4 weeks, followed by a decline toward baseline values with exposure cessation. A tendency for efficacy to fall with time, which may be associated with the development of neutralizing antibodies or tolerance, has been observed. The mean half-life of subcutaneous administration is 18 to 20 h, compared to 7 to 12 h following intravenous injection.[57]

As an increase in the carbohydrate content of Epo results in a longer plasma half-life and enhanced biological activity, more effective analogs have been developed.[58] Darbepoetin alfa, a novel erythropoiesis-stimulating protein or NESP (Aranesp®), is a glycosylation analog of rHuEPO that has been synthesized using DNA technology.[59] Its molecular weight is 38.5 kDa, and its total carbohydrate content is 52%. The amino acid sequence of NESP differs from that of native human Epo at five positions (Ala30Asn, His32Thr, Pro87Val, Trp88Asn, and Pro90Thr); this allows the attachment of additional oligosaccharides at asparagine residue positions 30 and 88.[60] Therefore, NESP contains five N-linked oligosaccharide chains and 22 sialic acid

residues, whereas native Epo contains three oligosaccharide chains and 14 sialic acid residues.

NESP binds to the Epo receptor in the same fashion as native Epo and induces intracellular signaling involving tyrosine phosphorylation by JAK-2 kinase and the intracellular molecules Ras/MAP-k, P13-k, and STAT-5. In comparing the pharmacokinetics of NESP and rHuEPO, the mean half-life for NESP is nearby two to three times longer than that of rHuEPO (25.3 and 48.8 h when administered intravenously and subcutaneously, respectively).[61] Despite a similar volume of distribution of nearby 50 ml/kg, the clearance of NESP is significantly reduced as compared to that of rHuEPO (1.6 ± 0.3 versus 4.0 ± 0.3 ml/h/kg). Dosage requirements of darbepoetin do not differ between the intravenous and subcutaneous routes of administration. The less frequent administration of darbepoetin alfa in comparison to the other epoetins may reduce drug costs in the long term, but the variability in dosage or dosage frequency required within a single patient is much higher.[61]

CERA (continuous erythropoietin receptor activator) is the latest erythropoiesis-stimulating protein that has become commercially available.[62,63] Administered at a considerably less frequent dosing (once every 3 or 4 weeks), CERA induces enhanced and sustained erythropoietic effects through continuous modulated stimulation of erythropoiesis and offers greater flexibility and convenience as compared to other conventional blood-boosting substances. Postulated mechanisms of action include a weaker binding to and a more rapid dissociation from the Epo receptor and an extended half-life in plasma, which has been reported to be seven-fold greater than that of standard epoetins.[62] At doses of 5 to 8 μg/kg, CERA increases reticulocytes and hemoglobin by 262% and 16 to 23 g/l (over 50% of patients had a response $\geq$20 g/l), respectively; these levels were maintained over the next 12 weeks.[62] Levels of sTfr also increase in a dose-dependent manner, whereas serum ferritin and serum iron levels decrease, reaching a nadir 5 to 10 days after administration before returning to baseline.[62] The mean elimination half-life is 133 to 137 h for both intravenous and subcutaneous administration. Side effects of CERA therapy are generally mild, and no serious adverse events have been reported thus far.[62] CERA is currently in phase III clinical trials.[62]

B. Regulation of Endogenous Erythropoietin Production

Approximately 90% of endogenous Epo is produced by the kidney in response to tissue oxygen sensors that register oxygen depletion. The recent discovery of a novel family of proteins called "hypoxia inducible factors" (HIF) has increased our understanding of the complex mechanism of response to hypoxia and Epo production that occurs when the human body has to deal with increased oxygen demand, such as in hard-working muscles.[64] Epo is the paradigm of oxygen-regulated genes controlled by transcription

factor HIF-1, well recognized as the key regulator of cellular and systemic oxygen homeostasis.[65]

Oxygen sensing is characteristic of all oxygen-dependent cells. The acute response to oxygen deprivation is carried out by a neurosecretory mechanism in glomus cells of the carotid body and in neuroepithelial bodies of the lung where the chemoreceptor cells sense the oxygen tension in the blood coming from the heart.[66,67] The cells' response to acute oxygen changes lasts only milliseconds and causes rapid cardiorespiratory adjustments. This is due to oxygen-sensitive cell membrane K+ channels of different kinds found in all hypoxia-responsive cells.[68–70] The reduction of oxygen tension leads to closure of K+ channels and to membrane depolarization and it is followed by extracellular Ca2+ influx and consequent transmitter release, which fires the response of the afferent nerve fibers.[71]

At present, the precise molecular mechanisms by which K+ and Ca2+ ion channels sense oxygen tension variation and thus activate the signaling pathways remain unclear, as findings depend largely on experimental conditions and on cell types used. The mechanisms underlying the chronic response to oxygen deprivation were discovered a number of years ago when a factor broadly expressed in hypoxic conditions was found by a group of researchers investigating Epo production regulation by body cells.[72] This factor, named HIF-1, was structurally characterized as a heterodimeric protein.[73] HIFs are transcription factors that modulate the activity of a variety of genes in conditions of relative low-oxygen availability. During hypoxia, the activity of two intracellular enzymes that promote the degradation of HIF-1a (asparaginyl hydroxylase and prolyl hydroxylase) is inhibited.[74] Therefore, HIF-1a binds to HIF-1b, crosses the nuclear membrane, and binds to intranuclear proteins, thereby promoting gene transcription. Genes controlled by the HIF pathway include those coding for proteins that stimulate erythropoiesis (namely, Epo) as well as those encoding glycolytic enzymes that produce additional energy in conditions of relative oxygen deficiency. These are pivotal mechanisms in the attempt to achieve improved aerobic performances.

C. Complications and Side Effects of rHuEpo Therapy

Despite its clinical effectiveness in selected clinical settings, concern has been expressed about complications and side effects of rHuEpo therapy.[75] In both controlled and uncontrolled clinical studies, the most frequent adverse events per 100 patient-years of exposure to epoetin delta were hypotension, muscle cramps, upper respiratory infections, headache, thrombosis, and hypertension.[32,75] An important concern is that the cardiovascular system of an athlete misusing erythropoiesis-stimulating proteins may be in jeopardy. In some clinical trials, thrombotic events such as myocardial infarction, cerebrovascular disease, transient ischemic attack, and venous thromboembolism occurred at a rate of 0.04 events per patient-year. In addition, there have been

occasional reports of serious or unusual thromboembolic events, including migratory thrombophlebitis, microvascular thrombosis, and thrombosis of the cerebral sinus, retinal artery, and temporal and renal veins.[76,77] Endothelial activation, alterations of blood rheology (namely, increased blood viscosity), increased systolic blood pressure at submaximal exercise, and platelet reactivity were acknowledged as important mechanisms involved in the thrombotic potential of Epo.[32]

Although an unequivocal causal relationship with rHuEpo therapy has not been established yet, polycythemic conditions characterized by hematocrit values exceeding 60%, as were anecdotally achieved in the mid-1990s when rHuEpo abuse was largely uncontrolled, when compounded by dehydratation, may have predisposed athletes to thromboembolic complications. Hypertension following rHuEpo therapy, mostly during the first 90 days of therapy, has been reported in clinical trials.[32] On occasion, hypertensive encephalopathy and seizures have been observed in dialyzed patients.[78,79] When data from all patients in the U.S. Phase III multicenter trial were analyzed, a trend of more reports of hypertensive adverse events occurring in patients displaying a faster rate of rise of hematocrit (greater than 0.04 in any two-week period) was observed.[80]

Far more threatening is the onset of red cell aplasia, a rare congenital or acquired condition characterized by an arrest in RBC production.[81,82] This rare but life-threatening complication has been observed predominantly with subcutaneous use of epoetin alfa produced outside the United States, after albumin was removed from the formulation.[57] Although the pathogenesis of this severe disorder has not been definitely elucidated, its development has been related to the interaction of multiple factors, including Epo formulation change and improper storage; these factors led to increased immunogenicity of the recombinant product, which could trigger the sudden development of neutralizing anti-Epo antibodies.[83] The adverse effects, withdrawals, and deaths were the same for NESP as for rHuEpo treatment, though there have been no reports thus far of antibody production in patients treated with NESP. Early recognition and withdrawal of Epo therapy is essential. Treatment with immunosuppressive therapy resulted in a suitable response, with resolution in several cases.[83] A less clinically threatening anemia, characterized by progressive erythroid marrow exhaustion, has been reported occasionally in patients receiving rHuEpo therapy.[84]

A final concern rises from the evidence that the Epo receptor pathway may be somehow involved in the growth, viability, and angiogenesis of malignant tumors. Increasing evidence has accumulated to show that Epo has pleotropic effects beyond regulation of the RBC mass. In the embryo, Epo acts a major regulator of vascular formation and organ growth, and Epo receptors are found in almost every embryonic tissue.[85] Several malignant human cell lines express Epo and Epo receptor mRNA regardless of origin, histological type, genetic characteristics, and biological properties and

respond to hypoxic stimuli by enhanced secretion of Epo.[86] On this basis, Epo seems to protect neoplastic cells from apoptosis and to stimulate them to proliferate, but these cells are unable to regulate the number of Epo receptor sites as occurs in erythroid differentiation.[87]

D. Antidoping Testing for Erythropoiesis-Stimulating Substances

The identification of Epo misuse in sport has been one of the most intricate challenges for laboratory medicine in the recent history of antidoping testing.[88] Once it became clear that misuse of Epo among endurance athletes was commonplace, the International Olympic Committee and most sport organizations attempted to develop strategies to identify cheating. Indeed, blood doping was not a novelty for laboratory medicine; however, before Epo became available, the problem was confined to a limited number of top athletes experimenting with blood transfusions. The numerous physiological and practical advantages of Epo encouraged the wide diffusion of this innovative doping technique, which rapidly emerged as a major issue for public health.[89] Despite the lack of reliable analytic tools, several sport federations, recognizing the seriousness of the problem, adopted a questionable strategy based on arbitrary thresholds for hemoglobin, hematocrit, or both. In particular, the International Ski Federation decided to pursue athletes with hemoglobin values above 175 g/l for men and 155 g/l for women, whereas the International Cycling Union disqualified athletes with hematocrit values exceeding 50% for men and 47% for women when reticulocytes were above 2%.[26] Although the evidence of increased or decreased erythropoiesis is not an unequivocal proof of blood doping, athletes exceeding such limits were suspended from competition for rather arguable reasons of health.[90] The evidence that such a strategy might also have penalized clean athletes encouraged the adoption of more suitable and elaborate approaches.

Over the last decade, both direct and indirect strategies for detecting rHuEpo were proposed, but no single approach was found to meet all the criteria required to identify cheating.[91] Most of these strategies relied on indirect markers of rHuEpo administration, including hematocrit, hemoglobin, macrocytic hypochromatic erythrocytes, sTfr, reticulocyte hematocrit, percentage of macrocytic RBCs, and serum Epo. A reference range study of key hematologic parameters in elite athletes, using ADVIA 120 technology, also examined two statistical models that are useful indicators of current (ON-model) or recently discontinued (OFF-model) rHuEPO abuse. The component variables of the ON-model are hematocrit, reticulocyte hematocrit, serum Epo, percent of macrocytic RBCs, and sTfr, while the OFF-model uses only the first three variables.[92] Although this method was successfully applied within antidoping campaigns, some pitfalls, such as the ability to detect misuse of novel erythropoiesis-stimulating proteins like epoetin delta, NESP and CERA,[93] the influence of training regimens,[94] altitude,[95] preanalytical

variability,[26,96] and the stability of blood specimens over time, have since emerged.[97,98] An alternate strategy is the use of the hematologic passport, which has been discussed before.[29]

As exogenous recombinant Epo is less negatively charged than the naturally occurring hormone, isoelectric focusing has emerged as a reliable approach for direct detection of rHuEpo and analogs in urine.[35] Electropherograms from urinary rHuEpo differ substantially from those of endogenous Epo, as the former produce four or five bands in the basic region, whereas the latter gives rise to up to 14 bands that overlap with and are parallel to those of rHuEpo in the basic region but that are also clustered in the acidic region.[99] A preliminary electrophoretic technique for discriminating rHuEpo from its endogenous counterpart in urine was described in 1995.[100] The method was improved by using isoelectric focusing that reduced the non-specific binding traditionally accompanying immunoblotting.[99–103] This isoelectric focusing technique detects rHuEpo in urine samples collected 3 days after nine doses of epoetin alfa with both 100% sensitivity and specificity. However, 7 days after the last dose, the overall sensitivity of the techniques falls to 50%.[99] The method is relatively inexpensive and may be implemented in different laboratories.

The indirect approach, based on multiple markers of enhanced erythropoiesis, appears to be a valid and reliable strategy associated with the urine confirmatory test. The combination of blood and urine tests represented the strategy implemented by the International Olympic Committee for detecting rHuEpo misuse at the Sydney Olympics. However, in analogy with other performance-enhancing drugs, during-competition testing has led to much wasted testing effort, as the pharmacodynamic properties of rHuEpo and its analogs discourage misuse near to or at the time of competition. Thus, direct testing methods, such as the rHuEpo urine test or the ON-model, will likely fail due to the almost complete elimination of rHuEpo before the test and the return to baseline of most biochemical parameters of erythropoiesis, unless international sporting federations use the information gathered to assist in targeted out-of-competition testing.[91] However, as the performance-enhancing effect is greater than variation of hematologic changes and persistence of rHuEpo in blood, the indirect OFF model, based on the expression of specific genes following rHuEpo administration, should partially overcome this limitation.[104]

Recently, novel reticulocyte indices have been investigated, especially in the setting of iron deficiency and functional iron deficiency during therapy with rHuEpo.[105] A specific concern regarding the analytical efficiency of the combined blood-urine strategy is the misuse of epoetin delta. Due to the innovative gene activation technology employed for its production, this recombinant Epo appears currently to be undetectable by this approach.[26]

Fortunately, Epo analogs appear to be more easily detectable, and some athletes were sanctioned for use of NESP at the 2002 Winter Olympics. Most

techniques measure the increased immunoactivity of NESP in serum following desialylation with neuraminidase, as measured by conventional immunoassays for Epo.[106] The method requires a small amount of blood, is simple to perform, is reliable (up to 100% sensitivity), and allows detection of NESP from 2 to 14 days after the last injection.

V. ALTITUDE TRAINING

Altitude training is a well-established "natural" and legal technique to improve endurance performance at sea level. At higher altitudes, the relative oxygen content of the air is diminished; after a sufficient period of time, the body responds with a complex series of biological and metabolic changes.[107] An understanding of the metabolic adjustment to altitude requires a review of human acclimatization and adaptation.[108] The term acclimatization is suited to relatively short exposures to altitude, such as short periods in training camps, while adaptation refers to changes occurring over generations under constant exposure. Some, but not all, of the main features of acclimatization and adaptation are similar. Acclimatization is basically heterogeneous, depending upon the type of environmental stress to which the athlete is exposed. Passive stresses, such as altitude and climate, are persistent and substantially uniform over time, whereas active ones like training regimen and diet are more variable and possibly mutable. Finally, compliance to excessive stress is highly sensitive to individual characteristics.

The duration of exposure to passive stress modifies the nature and resiliency of changes following stress removal. After exposure to moderate altitude, some initial changes occur in response to the changed conditions; as the exposure duration increases, the repertoire of changes expands and stabilizes. Therefore, the longer the body is in a fully acclimatized condition, the more habitual the changes become. Upon exposure to a passive stress, the body undergoes a hierarchy of responsive changes and eventually becomes fully acclimatized to the point that the changes become constant as well as permanent while residing in the environment. Full acclimatization is compromised by shorter periods of exposure; generally, the shorter the time spent at altitude, the less dramatic is the acclimatization and the changes that do occur are quite transient.

A. Physiology of Altitude Exposure

Several physiologic changes occur following acclimatization at high altitude. They can be divided into immediate (taking place over a few days) and long-term changes, which requires weeks to a few months. Immediate changes include decrease in maximum cardiac output, decreased maximum heart rate, increased erythropoiesis, increased excretion of base via the kidneys to restore acid-base balance, and an increase in the number of

mitochondria and oxidative enzymes in RBC that allow a more efficient unloading of oxygen to peripheral tissues.[106] The combination of altitude and time necessary to achieve significant athletic benefits, lasting 2 to 3 weeks after return from altitude, has been conventionally established at 2200 m for 4 weeks. This convention is supported by the practical evidence that a significant (77 to 92%) and stable (over 24 h) increase in Epo production occurs rapidly (within 6 h), once a threshold altitude of 2100 to 2500 m is reached. Below these altitudes, Epo increases are modest (24 to 30%) and unsteady, reaching a peak at 6 h after exposure.[109] Accordingly, 4 weeks of training at an altitude of 1740 m produce no changes in hemoglobin and a negligible increase in maximum oxygen consumption in highly trained athletes.[110]

The mechanism of the greater hematologic response to high altitude includes improved oxyhemoglobin desaturation that usually occurs as the oxygen partial pressure (pO_2) falls to the steep portion of the oxyhemoglobin dissociation curve.[110] Hypothetically, the higher the altitude, the greater the ergogenic benefits achieved. However, an upper limit of altitude should be set to limit the side effects of altitude exposure, such as chronic mountain sickness or Monge's disease, depression, headache, loss of appetite, sickness, and sleeplessness.[111–113] Again, the ideal period of living at altitude is uncertain, but it can be hypothesized from the physiology of erythropoiesis. The concentration of Epo in blood starts to increase from the first day of living at an altitude of 2500 m; by 2 weeks it stabilizes and after 4 weeks returns to the baseline.[114] Therefore, a period lasting not less than 3 to 4 weeks appears most appropriate.

Like astronauts who enter microgravity, people acclimatized to high altitude who descend to sea level undergo a rapid adaptive mechanism that modifies the excessive blood volume and RBC mass for the new environment. This physiologic process, called neocytolysis, results in selective hemolysis of young circulating erythrocytes.[115] Other physiologic situations associated with increased neocytolysis include the emergence of newborns from the hypoxic uterine environment and the descent of polycythemic high-altitude dwellers to sea level.[116] While both RBC production and survival remain normal, the RBC mass decreases up to 15% over a few days. Serum Epo levels are also profoundly suppressed on descent. Therefore, Epo dynamically suppresses erythropoiesis by initiating neocytolysis when serum levels are reduced below a nadir threshold.[117]

Thus far, the mechanisms involved in neocytolysis have not been fully elucidated. However, the substantial RBC mass reduction, associated with the unchanged rate of reticulocyte release and increased concentration of markers of erythrocytolysis (lactate dehydrogenase and bilirubin), provide evidence of a hemolytic process characterized by accelerated catabolism of young erythrocytes. Ultimately, the relative deficit of Epo production may shift the balance toward cell death, triggering apoptosis through multiple pathways.[118] The process may involve a modified interaction between

neocytes and reticuloendothelial phagocytes, particularly in the spleen, with likely roles for surface adhesion molecules and intermediary endothelial signals;[119] it may finally be prompted by endothelial cells communicating with macrophages to stimulate phagocytosis of this unusual cell subset.[120] Moreover, withdrawal of Epo production may induce activation of a subset of caspase-3, -7, and -8, which then cleaves the transcription factors GATA-1 and TAL-1 and finally triggers apoptosis.[121] The implications of neocytolysis may also extend to blood doping by surreptitious use of rHuEpo, as it may contribute to adverse consequences such as hyperkalemic arrhythmias.

A review of the current literature supports the benefits of altitude training, though there is a large variation in outcomes, possibly due to the heterogeneity of settings and periods of living at altitude or to the effects of active stresses and confounding variables (athletic discipline, training regimen, diet).[122] Since RBCs carry oxygen through the bloodstream, a substantial increase in the packed cells following altitude exposure allows more efficient oxygen delivery to the muscles at sea level, reducing fatigue and giving the athlete an edge. Altitude exposure is a popular practice among elite cyclists seeking enhanced performance at sea level, and some endurance athletes train at high altitude for precisely this reason.[123] With acclimatization, there is convincing evidence of decreased production or increased clearance of lactate in muscles, moderate evidence of enhanced muscle buffering capacity and tenuous evidence of improved mechanical efficiency of cycling.

Acclimatization to high altitude induces further central and peripheral adaptations that improve oxygen delivery and utilization.[124] Moreover, hypoxic exercise may increase the training stimulus, thus magnifying the effects of endurance training.[125] Acute exposure to moderate altitude enhances cycling performance on flat terrain because the benefit of reduced aerodynamic drag outweighs the decrease in maximum aerobic power. Several strategies have been proposed to optimize the acclimatization changes: live high and train high,[126] live high and train low,[127] and intermittent exposure.[128] The current literature indicates that continuous living and training at moderate altitude does not improve sea-level performance of high-level athletes.[123] Although oxygen delivery and utilization may be slightly improved, a lack of adequate training adaptation and/or decreased exercise intensity due to hypoxia can lead to a relative detraining effect that may overwhelm any advantage gained through altitude-induced acclimatization.[129] Therefore, acclimatization to a moderately high altitude, accompanied by training at low altitude (the so-called "living high-training low" theory), is likely the most effective variant of altitude training to improve sea-level endurance performance. Such an improvement is due to a wide series of physiological and biochemical adaptations, including a consistent improvement in maximal oxygen uptake, a rise of circulating Epo, the levels of which can be nearly double those of the initial sea-level baseline, and an increase of up to 10 g/l of hemoglobin concentration in blood.[130]

Despite promising results, some later investigations described wide interindividual variability in adaptive response and athletic performance after a traditional altitude training camp; two clusters athletes were observed—those who respond to altitude training and those who are actually "non-responders." It has been hypothesized that such individual variability may be accounted for by two mechanistic pathways: altitude-acclimatization response and training effects. Although the concentration of Epo in plasma rises significantly in both groups, the aggregate variation is definitely broad, ranging from 41 to 400%, and responders had a significantly larger and more persistent increase in absolute terms and when expressed as a percentage of sea-level baseline.[127] Such variation explains the further differences observed between responders and non-responders in the total RBC volume, which increases by 8% in responders but remains substantially unchanged in the non-responder cluster. Accordingly, post-altitude maximum oxygen consumption and endurance performance are both significantly increased in the responders, with no significant changes in the non-responders.[127] The reason for such a broad hematologic response is as yet unclear, but it is likely multifactorial and includes heterogeneity in hypoxic ventilatory drive, oxygen half-saturation pressure of hemoglobin, hypoxia-induced transcriptional regulation of Epo synthesis and production, Epo metabolism, and sensitivity of bone marrow stem cells to Epo and other growth factors.[116]

Some of these factors may be genetically inherited traits, associated with the physiology of endogenous Epo production.[131] As human Epo gene expression is regulated primarily at a transcriptional level in oxygen-sensitive cells,[132] and the oxygen-sensing system triggers production of HIF-1, these transcriptional and post-transcriptional mechanisms may play a pivotal role in the observed individual variability. However, regardless of the specific mechanism, it seems conceivable that non-responders require a greater hypoxic stimulus to induce an adequate erythropoietic response. The practical outcome of these studies is crucial. Biochemical or genetic screening of the erythropoietic and training velocity response to acute altitude may be realistically employed to minimize the number of non-responding athletes, optimizing on an individual basis both the logistic setting (appropriate altitude, type of exposure, training regimen) and the length of stay to achieve the greatest hematologic and athletic benefit. Revolutionary advances in biotechnology have allowed the identification of several genetic polymorphisms promoting sport advantages.[133] The early recognition of a young athlete's predisposition to a particular sport discipline, such as a polycythemic response, is regarded as an attractive prospect, allowing children and adolescents to select the most suitable discipline on an individual basis. Nevertheless, it may also open unpredictable scenarios that influence the athlete's prospects in both economic and recreational terms.[134]

Additional controversies have emerged from studies on the so-called "short-interval" or intermittent altitude training. Very short hypoxic episodes,

like sleep apnea, lead to a negligible and spurious increase in hemoglobin concentration that is mainly due to a hormonal-mediated decrease in plasma volume.[135] Brief and repeated (intermittent) exposures to high altitude might elicit a comparable effect in physiological measures associated with improved performance, but definitive evidence is lacking.[136] Although some studies on short-term intermittent hypobaric hypoxia (1.5 to 3 h/day for 2 to 3 weeks) demonstrated acclimatization and favorable hematologic adaptations,[137,138] others failed to demonstrate significant changes in hemoglobin, hematocrit, sTrf, reticulocytes, maximal oxygen uptake, mean power output, and overall endurance performance.[128,136,139–141] It is conceivable that discordant results may have arisen from heterogeneity in the study design (for example, substantial differences in the study population, and terms and conditions of exposure). Realistically, brief hypoxic triggers of Epo production by acute exposure of moderately trained subjects to normobaric hypoxia during a short-term training program consisting of moderate- to high-intensity intermittent exercise are not always associated with a concomitant activation of erythropoiesis and have no enhanced effect on the degree of improvement in either aerobic or anaerobic performance.[128,141] Taken together, current evidence suggests that short-term intermittent exposure to moderate hypoxia and hypobaric chambers may not be sufficient to improve aerobic capacity and to induce altitude acclimatization, whereas longer periods, up to 4 weeks, may be effective in eliciting hematologic modifications and improvement in endurance performance.

B. Artificial Altitude Environments

As altitude training has some logistical and biological disadvantages, scientists have investigated alternative techniques of simulating altitude exposure which may offer the same physiological benefits while minimizing the drawbacks. Since the introduction of the hypoxic training theory in 1930, several techniques have been developed, including regular high-altitude flights, training in altitude chambers, and training by inhalation of low-oxygen-gas mixtures.[142] These techniques were used primarily for altitude preacclimatization and for the treatment of clinical disorders including chronic lung disease, bronchial asthma, hypertension, diabetes mellitus, Parkinson's disease, emotional disorders, radiation toxicity, and prophylaxis of certain occupational diseases. The mechanisms at the basis of the beneficial effects of hypoxic training involve regulation of respiration, free-radical production, and mitochondrial respiration.[142] In general, hypoxic exposure can be divided into hypoxia at rest, with the primary goal of stimulating altitude acclimatization, and hypoxia during exercise, to enhance the training stimulus. Moreover, the hypoxic stimulus may be continuous or intermittent.[143]

A sleep chamber that enables endurance athletes to "train while they sleep" has been developed. The chamber simulates the reduced air pressure

of altitude environments equivalent to approximately 2000 to 3000 m. Athletes who use a hypoxic apartment typically "live and sleep high" in the hypoxic apartment but train at or near sea-level conditions, thus enabling them to achieve a similar advantage to those living at high altitude.[144] The appropriate use of the chamber (6 to 8 h a day for 2 to 3 weeks) produces substantial increases in serum Epo, reticulocyte count, and RBC mass (up to 23%), which, in turn, may lead to improvement in post-altitude endurance performance.

Altitude simulation technology has continued to evolve. The hypoxic tent system is one of the first alternatives to the sleep chamber. The hypoxic tent system creates a hypoxic environment via a patented air separation unit that continually pumps low-oxygen content air. Inside the tent, the total pressure is unchanged, but the pO_2 is reduced, allowing athletes to obtain the advantages of altitude training at any location.[145] Unlike the constant hypoxia at higher altitudes, the intermittent hypoxia generated by the hypoxic tent system promotes gradual biological adaptation to yield better performance not only in a low-oxygen environment but also in normoxic conditions, such as at sea level. Earlier studies on simulated altitude by normobaric or hypobaric hypoxia convincingly demonstrated increases in Epo of a magnitude similar to that observed in response to a real altitude exposure of ~2.500 m.[126,145] Hypoxic sleeping devices include the Colorado Altitude Training Hatch (hypobaric chamber) and the Hypoxico Tent System (normobaric hypoxic system), which simulate altitudes of up to approximately 4575 m and 4270 m, respectively. Little information is available so far on the efficiency of these devices on increasing erythropoiesis, maximal oxygen uptake, and athletic performance. Hypothetical benefits are based on the evidence that brief exposure to hypoxia (1.5 to 2.0 h) is sufficient to trigger RBC production.[144]

An alternative technique to simulate altitude is breathing hypoxic, normobaric gas mixtures. In these conditions, athletes breath air with progressive decreases of the fraction of inspired oxygen (from 12.2% to 10.0%).[146] Two main approaches to this technique were investigated: continuous inhalation of a hypoxic mixture containing 12% of oxygen for 30 min, and intermittent exposures of 5 to 7 min to steady or progressive hypoxia, interrupted by equal periods of recovery with normoxic respiration.[147] Intermittent hypoxic exposure resulted in pronounced increases in heart rate and oxygen utilization by the end of the hypoxic session. Supplemental oxygen may be used during high-intensity workouts at altitude to simulate either normoxic or hyperoxic conditions. This method is a modification of the "high-low" strategy, since athletes live in a natural terrestrial altitude environment while they train at sea level breathing supplemental oxygen. Limited data regarding the efficacy of hyperoxic training suggests that high-intensity workouts at moderate altitude and endurance performance at sea level may be enhanced when supplemental oxygen training is employed at altitude over several weeks.[144]

In general, the efficacy of simulated altitude is achieved by an increased erythropoietic response and improved performance in athletes of endurance sport disciplines. Although earlier evidence in untrained subjects was promising, it is as yet questionable whether elite athletes, who may be closer to the maximal structural and functional adaptive capacity of the respiratory system, may achieve real gains from simulated altitude devices. Most clinical discrepancies are due to the marked heterogeneity in exercise, environmental conditions, nutritional therapies, duration, repetition rate, and intensity of the artificial hypoxic stimulus.[146] If a distinct advantage is needed in endurance events, the use of high-altitude simulators would appear less risky than other forms of blood doping. However, there are some ethical and clinical concerns among coaches, athletes, and the scientific community that the use of these expedients may be unsafe and unethical for use in sports. Just as training preparation that gives an athlete a technological or athletic advantage that is too expensive or innovative for most other competitors to use is considered unfair,[3] simulated altitude is currently considered unethical. Although this position has been questioned, as the biological changes induced by altitude simulation are almost indistinguishable from those of real exposure and training at altitude, simulated altitude, called "holistic blood doping," ultimately reproduces most of the physiological and pathological effects of rHuEpo and is now considered an illegal mean of performance improvement.[148] Such a conclusion is reinforced by the onset of a wide series of symptoms, such as anorexia, insomnia, hyperventilation, headache, dizziness, and deterioration in higher cerebral functions, that characterize the initial phase of induction.[149] Finally, it is recognized that some athletes experience considerable reduction in the ability to train at the appropriate intensity.

VI. BLOOD SUBSTITUTES

There is a long history of science seeking to develop artificial substitutes for damaged body parts. While some body part, such as teeth and limbs, may be replaced by "imitations" without major loss of functionality, the development of a substitute for RBCs has proven elusive to date. Historically, blood loss from critical surgery or trauma has been treated with either volume-replacing fluids or transfusions. The use of transfusions in acute anemia is well established, but clinical and technical problems, along with an increasing demand and a constantly decreasing supply for blood, limit the use of elective allogeneic transfusions in clinical practice.[150] The use of autologous blood for elective surgery, in combination with rHuEpo, has proven to be a successful and cost-effective approach for minimizing the use of allogeneic blood. However, such a strategy cannot address all the problems of decreased oxygen delivery, especially when the need for oxygen-carrying supplementation is not anticipated, for example, for organ preservation and for some

forms of acute anemias (emergency resuscitation). Finally, blood substitutes meet the requirements of patients who cannot receive donor blood because of religious beliefs.

Progress in chemical and biotechnological research has allowed the development of a novel approach to this issue, in the form of temporary oxygen carriers, widely known as "blood substitutes." The blood substitutes currently available are chiefly polymerized hemoglobin solutions or hemoglobin-based oxygen carriers (HBOCs) and perfluorocarbons (PFCs).[151,152] Alternatives include artificial RBCs, in which hemoglobin and supporting enzyme systems are encapsulated into liposomes. Major clinical advantages of these substitutes include sterilization of viral and bacterial contaminants, room temperature storage, longer shelf life, and absence of RBC antigens.[153–155]

A. Perfluorocarbon Emulsions

PFCs are inert, water-insoluble, synthetic aromatic or aliphatic compounds, consisting primarily of carbon and fluorine substitutions for most hydrogen atoms. PFCs are substantially clear and colorless liquid emulsions that are heterogeneous in molecular weight, surface area, electronic charge, and viscosity; their high content of electron-dense fluorine atoms results in little intramolecular interaction and low surface tension, making such substances excellent solvents for gases, especially oxygen and carbon dioxide.[151] Some of these molecules can dissolve 100 times more oxygen than plasma. PFCs are naturally hydrophobic and need to be emulsified to be injected intravenously. Recently, sophisticated technology has allowed the generation of stable emulsions that contain tiny particles with a median diameter $<0.2\ \mu m$ and that display a very low molecular weight (about 500 Da).[156,157]

Considerable effort has focused on ensuring the long-term stability of ready-to-use, concentrated PFCs. Since PFCs dissolve rather than bind oxygen, their capacity to serve as a blood substitute is determined principally by the pO_2 gradients in the lung and at the target tissue. Therefore, their oxygen transport properties differ substantially from those of whole blood and, especially, from those of RBCs. Erythrocytes exhibit a sigmoidal oxygen dissociation curve, whereas PFCs are characterized by a linear relationship between pO_2 and oxygen content (Figure 1).[158] At a conventional ambient pO_2 of 135 mm Hg, the oxygen content of 900 ml/l perflubron is less than 50 ml/l, whereas an optimal oxygen content of 160 ml/l, which is still lower than that of whole blood in normal conditions, can be achieved only by a pO_2 greater than 500 mm Hg. In practice, at a conventional alveolar pO_2 of 135 mm Hg, PFCs will not be able to provide sufficient oxygenation to peripheral tissues.[158,159] Owing to this limitation, optimization of the oxygen transport capacity can be achieved only through a substantially raised arterial pO_2 (i.e., ventilation with 100% oxygen), which appears unsuitable and most unlikely outside a controlled hospital setting. Moreover, prolonged oxygenation may

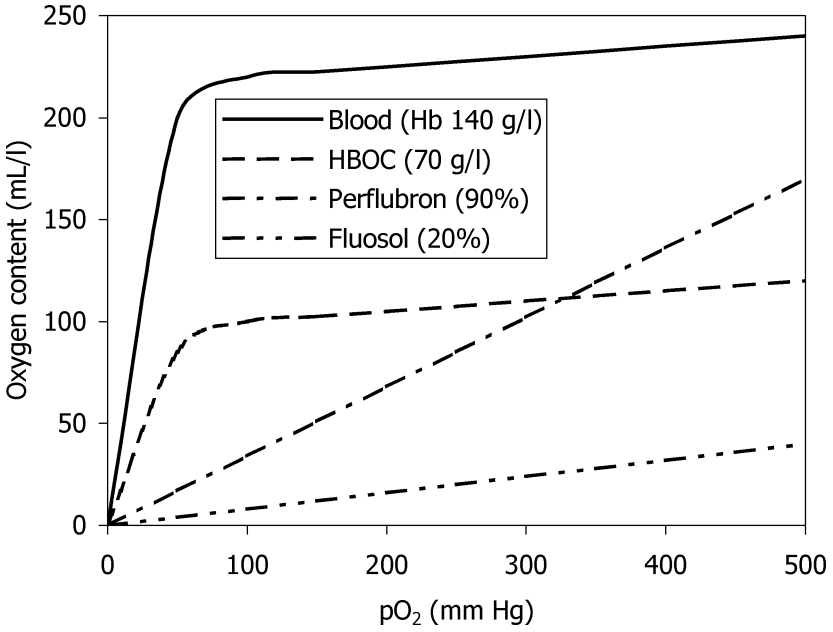

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FIGURE 1 Comparison of oxygen capacity of whole blood, HBOC and two PFC blood substitutes versus oxygen tension.

lead to oxidative stress and tissue damage and may trigger potentially adverse genetic effects.[160]

Owing to their small size, PFCs efficiently perfuse the microcirculation where erythrocytes may not flow. In tiny capillaries, PFCs produce the greatest benefit, as they increase local oxygen delivery much more efficiently than would be expected from the increase in oxygen content in larger arteries.[161] In addition, as gases are in the dissolved state within PFCs, the pO_2 in the microcirculation may be considerably increased, thereby promoting an extremely efficient oxygen delivery to peripheral tissues. PFCs undergo a rapid and atypical metabolism. After initial uptake by the mononuclear phagocytic system, the emulsion particles are rapidly degraded, and the PFCs re-enter the blood stream mainly bound to blood lipids; they are finally excreted by the lungs via exhalation. The half-life of PFCs depends on the chemical composition and appears strongly dose dependent, ranging from 2 to 6 h.[162] The first demonstration of the clinical efficacy of PFCs was provided in mice in 1966.[163] Since the mid-1980s, improvements in both oxygen capacity and emulsion properties of PFCs have led to the development of second-generation PFC-based oxygen carriers; two PFC products are currently being tested in phase III clinical trials.[159–164]

The safety of oxygen substitutes has been an objective for nearly a decade as effective replacements for blood transfusion have become available. Some threatening side effects were reported in clinical trials using PFCs. A delayed

and transitory "flu-like" syndrome was occasionally described; symptoms consisted mainly of back pain, malaise, flushing, and a transient fever lasting several hours.[162] As PFCs are cleared by cells of the reticulo-endothelial system, and the febrile response was blocked by ibuprofen or dexamethasone, it is conceivable that this syndrome was elicited by the release of inflammatory cytokines, namely, interleukin 6.[165,166] PFCs affect cell activation in a non-specific manner, as measured by tyrosine phosphorylation, likely by interfering with transmembrane signal transduction.[167] An additional threat is the effect of PFCs on platelet function. Aggregation by collagen, ADP, or arachadonic acid appears substantially inhibited in a dose-dependent fashion in *ex vivo* porcine platelets following infusion of perflubron emulsion at a dose of 3 ml/kg.[168]

Given some elite athletes' innate inclination to experiment with novel doping strategies, it is speculated that some have used artificial oxygen carriers, but no clearly positive cases have been defected so far. There are several issues regarding efficacy and clinical safety of blood substitute administration in athletes. Scientific data concerning the performance benefits are inconclusive. Although the use of HBOCs and PFCs may theoretically produce an athletic advantage, the biochemical characteristics (linear relationship between dissolved oxygen and pO_2), the form of administration (intravenous), the need for high oxygenation, the short half-life (up to 6 h) and the side effects still hinder the diffusion of this rather atypical form of blood doping. This conjecture is reinforced by the clinical evidence that in surfactant-deficient lungs, single and multiple applications of PFCs only transiently improve oxygenation, whereas continuous infusion is needed to provide the best gas exchange.[169] Thus, although both rHuEpo and artificial oxygen carriers persist only for a short time in the circulation, the latter would have to be used by athletes just before the competition to achieve a substantial performance advantage. Of course, as PFCs would require a high-oxygen environment to function, an athlete carrying his oxygen tank would be easily noticed.

Despite these clear limitations, international sporting federations have been commendably proactive in adding this category of compounds to their banned substance lists. Blood screening and measurement of PFCs is as yet a major challenge, as methods are based on specific PFC analyzers and flow-measuring devices. Commercial instruments for PFC assays are expensive and unsuitable for clinical use.[170] However, as PFCs are mostly cleared by the lungs, they can be measured in expired air with a simple, on-line thermal detector analyzer,[171] by an infrared absorption technique,[172] or by absorbers containing specific PFC-absorbing aluminosilicates (zeolites).[173]

B. HBOC

Hemoglobin is naturally suited to bind, carry, and deliver oxygen when encased by the RBC membrane; once removed, it becomes dysfunctional and

rapidly dissociates. The administration of free hemoglobin to humans is unsuitable; dimers of about 32 kDa produced by dissociation are cleared by the kidney where they may accumulate, generate renal obstruction and oxidant injury or necrosis, and trigger renal failure.[174,175] Therefore, hemoglobin must be stabilized before it can be safely infused. HBOCs are intra- and/or inter-molecularly "engineered" human or animal hemoglobins, optimized for oxygen delivery and longer intravascular circulation. Several approaches have been attempted to stabilize and modify the hemoglobin molecule: polymerization of human (PolyHeme) or bovine (HBOC-201) hemoglobin with glutaraldehyde or raffinose (Hemolink), pyridoxylation, conjugation with polyethylene or malemide-polyethylene glycol (MP4), and the cross-linking of the subunits by diaspirin (HemAssist) and oxidized mono/di/tri/poly saccharides.[175–177]

As well, enzyme cross-linked poly-hemoglobin- and hemoglobin-containing vesicles (nano-dimension artificial RBCs) have recently been developed.[175] Novel recombinant polymeric human hemoglobins (rHb1.1 and rHb2.0), such as those containing alpha-human and beta-bovine chains derived from genetically engineered *Escherichia coli* or those with amino acid substitutions in the central cavity of the molecule, are currently under development.[175,178,179] Most of these synthetic compounds exhibit low oxygen affinity, high cooperativity, an enhanced Bohr effect, and a slower rate of autoxidation of the heme iron, which finally results in a more efficient oxygen delivery.

The presence of 2,3-diphosphoglycerate within erythrocytes maintains the normal affinity of hemoglobin for oxygen. As erythrocyte-free hemoglobin loses this interaction, unmodified human HBOC solutions have a very high oxygen affinity which compromises their function. Chemical methods developed to overcome this problem have resulted in carriers that effectively release oxygen at the physiological pO_2 of peripheral tissues (Figure 1).[180] A common feature of all HBOCs is their resistance to disaggregation when dissolved in infusion media, which contrasts to the native hemoglobin property of natural dissociation under non-physiologic conditions.

First-generation HBOCs were developed principally to serve as oxygen carriers and as a substitute for erythrocytes in situations of acute and clinically threatening blood depletion, such as peri-operative use.[176] Depending on the type of modification used to stabilize the protein, second-generation HBOCs were considered to be useful in other clinical settings, such as enhancement of radiation therapy and nitric oxide scavenging.[181] Third-generation HBOCs, based on microencapsulation of hemoglobin and RBC enzymes either in liposomes or in biodegradable nanocapsules, appear so far to be the most promising products and are currently under evaluation in phase II and III clinical trials.[175,182,183] Following administration of a loading dose and a continuous infusion of polymerized bovine hemoglobin, HBOCs' elimination is a linear, first-order process without renal contribution to excretion.

The plasma half-life ranges from 12 to 48 h for cross-linked and surface-linked hemoglobin, respectively.[174]

HBOCs may hypothetically supply more benefits to athletes than those yielded by the equivalent hemoglobin provided by an erythrocyte infusion. Recent investigations have shown that HBOCs are not only simple erythrocyte transfusion substitutes, but highly effective oxygen donors in terms of tissue oxygenation. Additional effects include increases in serum iron, ferritin, and Epo;[184] up to 20% increased diffusion of oxygen and improved exercise capacity;[185] greater carbon dioxide production; and lower lactate levels.[186] Therefore, the possibility of athletes using HBOCs should be acknowledged.

There are several concerns about the therapeutic and unfair use of HBOCs. Phase II clinical trials and biological studies suggest that resuscitation with HBOCs, in lieu of stored RBCs, attenuates the systemic inflammatory response invoked in the pathogenesis of multi-organ failure. Specifically, HBOCs obviate stored RBC-provoked neutrophil priming, endothelial activation, and systemic release of interleukins 6, 8, and 10.[183] Some HBOCs are in clinical phase III trials, but no product has yet achieved market approval in the United States, Europe, or Canada,[175] as free hemoglobin and many forms of modified hemoglobins display strong vasoconstrictor reactivity due to the scavenging of endogenous nitric oxide, a powerful vasoactive agent released from endothelial cells.[187] Therefore, administration of HBOCs may generate microvascular permeability and failure, causing systemic and pulmonary hypertension. Additional threatening side effects include gastrointestinal dysfunction characterized by increased tone of the intestinal sphincters, marked flatulence and meteorism, renal toxicity,[174] and alteration of some biochemical and hematologic parameters, including increase in liver enzymes and alteration of platelet function. Finally, HBOCs derived from human or animal hemoglobins may carry infections agents.[188]

Presently, there are no commercially marketed blood substitutes available to athletes for blood doping. If one of the HBOCs becomes available, red (hemoglobin-colored) plasma or urine would make it readily detectable. However, HBOCs recently have been included within the International Olympic Committee and the World Anti-Doping Agency lists of substances and methods prohibited in sports. A variety of methods for separation, detection, quantification, and confirmation of HBOCs have been developed to deter athletes from doping with these compounds, though the direct visual screening of plasma discoloration appears so far the most suitable approach, with detection limits of less than 1% HBOC in plasma.[189] Alternative confirmatory techniques are based on electrophoresis,[190] liquid chromatography tandem-mass spectrometry,[191] and size-exclusion high-pressure liquid chromatography,[192] which are able to separate native hemoglobin from modified hemoglobin molecules characterizing HBOCs; in tandem with the electrophoretic screening, high-pressure liquid chromatography

and mass spectrometry meet the criteria for use in antidoping control settings.

C. Allosteric Modulators of Hemoglobin

Allosteric ("different shape," from allos = other and steric = solid or space) activators or inhibitors of proteins are substrates designed to alter the affinity of a protein for a ligand or an enzyme for its substrate.[193] Allosteric modulators usually bind non-covalently to the enzyme at regulatory sites that are distant from the catalytic or active sites.[194] The improvement of oxygen delivery to hypoxic tissues by a decrease in the oxygen affinity of hemoglobin has been a major aim in recent years, because this may reduce the consequences of anemia and/or improve tissue oxygenation in cases of decreased blood perfusion.[195] Accordingly, several allosteric modulators of hemoglobin have been synthesized; among these, (RSR) 2-[4-[(3,5-dichlorophenylcarbamoyl)-]methyl]]-2-methylpropionic acid 13 and RSR 4, both analogs of the drugs clofibrate and bezofibrate, so far to be appear the most effective compounds. They both decrease hemoglobin oxygen affinity through stabilization of deoxyhemoglobin, shifting the oxygen dissociation curve to the right and thus reproducing the effect of the natural allosteric effector of hemoglobin, 2,3-diphosphoglycerate.[196] This is commonly reported as a change in p50, the partial pressure at which hemoglobin is 50% saturated.[197] Therefore, RSR 13 and RSR 4 powerfully promote oxygen unloading from erythrocytes to hypoxic tissues,[198] and, on the basis of preclinical data, they may increase pO_2 in both normal and tumor tissue by about 5 to 15 mm Hg.[199] An additional series of novel molecules, including JP7, RSR 46, and pyruvate kinase inhibitors, are under development.[200–202] RSR 46 and JP7 could be employed to treat acute hypoxia, whereas PK inhibitors are designed to treat chronic hypoxia.

The spectrum of clinical applications of allosteric modulators is rather broad. Since these synthetic compounds increase tumor pO_2 and reduce tumor hypoxic fraction, they have been developed to maximize the effectiveness of radiation therapy. They also may be useful in acute ischemic disorders, such as acute coronary syndrome and brain ischemia, as they improve myocardial oxidative metabolism and contractile function in models of myocardial ischemia and increase brain pO_2, thus reducing neuronal cell death following cerebral ischemia. The RSR 13 half-life in healthy subjects is approximately 3 to 6 h, and the minimally effective therapeutic dose is nearly 50 mg/kg. At this dosage, RSR 13 would increase the p50 by approximately 2 to 4 mm Hg for approximately 2 h and would require an infusion volume of 350 mL administered intravenously over approximately 45 min.[203] Renal elimination has been suggested as the primary route of RSR 13 clearance.

Although RSR13 has never been tested in humans for sport performance enhancement, its biological effects make it a potentially effective

performance-enhancing agent for endurance athletes. Therapy with RSR 13 in patients with neoplasia is generally well tolerated; however, the potential spectrum of side effects in healthy individuals is cause for concern. Nearly one-third of patients undergoing RSR 13 therapy experience significant side effects, including headache, nausea, mucosal irritation, hypoxemia, allergic reaction, and transient renal dysfunction, characterized by increased levels of serum creatinine. In addition, RSR 13 administration in excess of the therapeutic dose without supplemental oxygen administration can result in significant arterial desaturation and grade 3 hypoxemia, which could lead to impairment of athletic performance.[199,202]

Another concern emerged when, after having been submitted to the U.S. Food and Drug Administration in December 2003, RSR 13 missed the primary endpoint in the pivotal trial and thus failed to be recommended for approval by the Food and Drug Administration Oncologic Drugs Advisory Committee for the year 2004. An athlete would not be able to benefit from use of RSR 13 if laboratory testing could detect its usage. A novel gas chromatography/electron impact ionization mass spectrometry technique for detection of RSR 13 in human urine up to 36 h after intravenous administration has recently been proposed.[204]

VII. SUPPLEMENTATION THERAPIES

The use of dietary supplements is commonplace among amateur and elite athletes, although evidence of athletic benefit is controversial. Although there is a the lack of reliable scientific evidence, many supplements may actually be detrimental to both performance and health, especially when taken in high doses for prolonged periods. Exposed to advertisements focusing on the supposed ergogenic benefits, some athletes aiming to gain a competitive edge have turned from banned substances toward nutritional supplements without considering the potentially harmful side effects.

A. Iron Supplementation

People who engage in physically active lifestyles are frequently targets of advertisements proclaiming the need for vitamin and mineral supplements. Owing to the potential risk to health and to the unfair of improvement athletic performance, reliable methods developed to identify the misuse of most doping techniques have allowed their inclusion within antidoping panels. However, only minor attention has been given to the potential risks to health of permitted supplements, which are considered relatively safe and are not included in antidoping testing. Such a concern is reinforced by a review of the 2005 version of the world antidoping code, annually issued by the World Anti-Doping Agency: "The use of any drug should be limited to medically justified indications."[205] Iron, an ubiquitous metal of vital importance

to normal physiologic processes, is one of these substances.[206] Although the risk of iron deficiency or depletion in athletes is limited to specific situations, namely the female sex, increased training stress, increased iron losses, and poorly balanced vegetarian diets, iron-supplementation therapy is commonplace in athletes to counterbalance physiological or pathological anemia and to prevent physiological dysfunction.[207] Iron supplementation may be initiated on the basis of unproven evidence of performance improvement or it may accompany the administration of erythropoiesis-stimulating substances, as iron bioavailability is crucial in enhancing the efficacy of rHuEpo.[208]

In 2001 the Institute of Medicine of the National Academy of Sciences set an estimated average requirement, recommended dietary allowance, adequate intake, and tolerable upper-intake levels for iron for healthy people.[209] Accordingly, a recent pronouncement from the Australian Institute of Sport affirms that iron supplementation may be initiated by a sports physician only after an appropriate diagnosis of inadequate iron status, as a part of an iron treatment program that also involves a dietary review and appropriate assessment of iron losses.[210] While an athlete who is iron deficient may not be overtly anemic, he/she may have a hemoglobin level that is less than optimal. Correcting iron deficiency anemia is not blood doping and may improve an athlete's performance. However, taking excessive oral iron supplements cannot increase hemoglobin above the reference range and may occasionally result in iron overload, which is associated with serious metabolic risks.[211–220]

While transferrin saturation is considered an indicator of an underlying genetic defect associated with primary hemochromatosis, the linear relationship between body iron load and serum ferritin makes the measurement of ferritin concentration a suitable approach to assess the total body iron content and detect potential misuse,[221] especially in endurance athletes.[213] Serum ferritin measurement, ordinarily performed in athletes entering elite training programs to identify hematologic and iron-related abnormalities,[210] allows early recognition of iron overload,[216] which may persist for a long time in most athletes after retirement from competition and cessation of excessive iron supplementation. Therefore, the emerging concern of health risks associated with indiscriminate iron supplementation in athletes requires a complete revision of the antidoping panel.

B. Cobalt Chloride Administration

The induction of a hypoxic condition generates a wide series of adaptative responses, which are also mediated through endogenous gene modulation in the HIF pathway. In an animal model, the administration of cobalt chloride promotes selective activation of HIF-1 signaling through increased DNA binding activity of HIF-1a.[222,223] In clinical practice, cobalt chloride is administered to treat some forms of anemia.[224] There is as yet no evidence

of cobalt chloride administration to athletes. However, given some athletes' inclination to experiment with innovative and unfair performance-enhancing techniques, cobalt chloride administration may be attemped to boost endogenous Epo production or to support unfair rHuEpo administration, as cobalt is currently not included on the list of banned substances.[225]

Although definitive information on metabolism and health risks of inorganic cobalt salt administration is not available, it accumulates to a greater extent in liver and kidney, promoting organ damage and dysfunction due to enhanced oxidative stress.[226,227] Excessive cobalt administration may also negatively influence thyroid activity.[228] Owing to this severe and unpredictable side effect, cobalt chloride administration could be a serious threat to the sport community and athletes' health. This concern has prompted the introduction of cobalt salt testing in revised antidoping panels.[225]

VIII. GENE DOPING

Several potential scenarios for blood doping have succeeded over the past decades. Nowadays, these effective performance-enhancing techniques may have been replaced by gene doping.[229–232] Since the early 1990s, experimental and revolutionary gene therapies have become available for the treatment of inherited pathologies and single-gene disorders such as hemophilia and hemoglobinopathies. Unfortunately, these promising therapies may embrace sports medicine and be used to boost or optimize athletic performance. Thus, gene or cell doping is currently defined by the World Anti-Doping Agency as the "non-therapeutic use of genes, genetic elements and/or cells that have the capacity to enhance athletic performance."[231]

Epo, human growth hormone, insulin-like growth factor-1, peroxisome proliferator-activated receptor-delta, and myostatin inhibitor genes have been identified as primary targets for doping.[233] Thus, gene doping aimed at stimulating erythropoiesis and improving aerobic performance is currently seen as an ominous development in the sphere of blood doping. The recent elucidation of the genetic mechanisms at the basis of the erythropoietic response to hypoxia has persuaded the pharmacological industry to develop novel agents that target the HIF pathway.[230] The biochemical or genetic manipulation of HIFs is, therefore, an alternate and potentially effective approach for unfair improvement of aerobic performance, with little chance of testing positive within current antidoping controls.

Several aspects of gene doping predict easy success for this approach. Transfection of genes identical to those naturally occurring in the human genome negate the risk of positivity in antidoping testing, as gene doping is virtually undetectable by traditional laboratory techniques. Moreover, gene doping exhibits long-lasting effects that abolish the need for repeated administration of pharmacological agents; this may persuade athletes to consider

it a more attractive, safer, and easier option than traditional doping. The systemic delivery of Epo in animals by intramuscular injection of adeno-associated virus vectors, already accomplished experimentally, produced supra-physiologic levels of Epo along with a polycythemic response.[234–237] Recently, a tissue protein factory based on dermal cores, implanted autologously in 10 patients with chronic renal failure, increased Epo significantly to therapeutic levels, which could be maintained for up to 14 days before declining because of a dermal infiltrate of CD8 cytotoxic T cells.[238]

Although there is as yet no definitive evidence of genetic manipulation in athletes, gene doping is a serious health concern as gene therapy is not safe from side effects, most of which are not predictable and are potentially deleterious. The onset of severe complications of gene transfection, such as insertional oncogenesis following inactivation of tumor suppressor genes or activation of proto-oncogenes,[239] propagation and recombination of retroviruses or adenoviruses vectors,[240] and potential humoral and cellular immune responses against transgenic proteins,[241] could be ethically justified for the treatment of patients with serious, life-threatening disorders,[242] but it is an unacceptable risk for healthy individuals who seek to enhance athletic performance. Such treatment is virtually impossible to reverse; early benefits in terms of improved athletic performance could later turn into serious complications, from thrombosis to cancer. In the specific case of Epo gene therapy, the administration of recombinant adeno-associated virus serotype 2 vector containing feline Epo cDNA resulted in pure RBC aplasia or pathologic erythrocytosis, which could not be abolished by injection site removal.[237] The potential manipulation of the HIF pathway poses serious risk to health, as HIFs target genes that may up-regulate cancer growth and spread.[230] Finally, besides health concerns, "gene cheating," as does doping in general, contravenes the ethics of sports and influences outcomes of competitions that frequently result in considerable economical benefit.

The characteristics and originality of gene doping have generated new and major challenges for detection that will require molecular techniques that recognise Epo transgenes or gene transfer vectors. However, most of these methods require invasive procedures that are unsuitable for screening large numbers of athletes. Indirect methods, based on hematologic or molecular profiling, may be better suited for screening, and athletes who test positive at this first step would then be required to submit to confirmatory scrutiny by direct molecular analysis.[243] The identification of potential links between changes in human biological profiles[244] and molecular insertion site analysis, which uses the transgene as a tag to identify neighboring cellular sequences, should be a powerful tool to help antidoping strategies. This technology appears crucial for determining the extent to which different types of vectors are attracted to particular areas of the genome, and, combined with functional studies, such investigations will provide an important basis for future developments.[245]

IX. CONCLUSIONS

Owing to the continuous progress of technology, biology, and sport medicine, blood doping is still a controversial issue that will remain a major challenge for years to come. Blood doping is unfair and not always detectable by current antidoping strategies; the potential risks for health outweigh any potential benefit. As long as we proceed further in the understanding of the intricate molecular mechanisms that regulate erythropoiesis, innovative and revolutionary therapeutic resources will become commercially available. As occurred earlier for other performance-enhancing substances, the side effects of these therapeutic agents used by athletes will become apparent in the future. The long history of doping in sports teaches us how eagerly people will reach for premature technology, with little fear of potential side effects or complications. Athletes may take advantage of many innovative therapies to improve athletic performance, and genetic doping is presently the most attractive method. Unfortunately, the hypothesis of genetically altered athletes is not simply imagination, and future scenarios are disturbing: athletes shattering phenomenal records and testing negative for traditional performance-enhancing drugs. Yet, something about the athletes may be different: their genes may have been altered. Scientists and sports federations should be aware of this problem. Heading off what will be an inevitable problem in the near future, the International Olympic Committee, the World Anti-Doping Agency, and international sport federations have recognized and banned genetic enhancement in a timely manner,[231] though effective and reliable approaches to unmask cheating are still to come.

Staying ahead in the fight against the use of blood doping involves staying ahead in basic and clinical research. There is growing evidence of unsuspected and previously undetected agents being used, and the potential list appears to be endless. Awareness of this emerging problem involves the need for cooperation among manufacturers, sport federations, antidoping agencies, and laboratory professionals to ensure that methods for the detection of putative doping agents are available at the time of product release. Scientists should be aware that the continued integrity of sports competition and athletes' health both depend on their ability to outpace the efforts of dopers; this calls for on-going development and the application of reliable detection strategies.

The World Anti-Doping Agency Code sets out the philosophical and practical basis for the antidoping regime in all sport federations that are signatories.[205] Any test implemented must now satisfy strict criteria that includes validation and replication in laboratories other than the one that created the test and peer review and expert panel review of the methodology. This innovative approach is likely to set off a sequence of legal consequences that could have many outcomes, as positive testing within antidoping campaigns might result in court action against an athlete who is found guilty.

REFERENCES

[1] Collins M. Sport, physical activity and social exclusion. *J Sports Sci* 2004; **22**: 727–740.
[2] Cronin J, Sleivert G. Challenges in understanding the influence of maximal power training on improving athletic performance. *Sports Med* 2005; **35**: 213–234.
[3] Verroken M. Drug use and abuse in sport. *Baillieres Best Pract Res Clin Endocrinol Metab* 2000; **14**: 1–23.
[4] Gaudard A, Varlet-Marie E, Bressolle F, Audran M. Drugs for increasing oxygen and their potential use in doping: A review. *Sports Med* 2003; **33**: 187–212.
[5] Rose AJ, Richter EA. Skeletal muscle glucose uptake during exercise: How is it regulated? *Physiology (Bethesda)* 2005; **20**: 260–270.
[6] Stirling JR, Zakynthinaki MS, Saltin B. A model of oxygen uptake kinetics in response to exercise: Including a means of calculating oxygen demand/deficit/debt. *Bull Math Biol* 2005; **67**: 989–1015.
[7] Warburton DE, Gledhill N, Quinney HA. Blood volume, aerobic power, and endurance performance: Potential ergogenic effect of volume loading. *Clin J Sport Med* 2000; **10**: 59–66.
[8] Sandler SG, Yu H, Rassai N. Risks of blood transfusion and their prevention. *Clin Adv Hematol Oncol* 2003; **1**: 307–313.
[9] Ramsey G. Managing recalls and withdrawals of blood components. *Transfus Med Rev* 2004; **18**: 36–45.
[10] Cobain TJ. Fresh blood product manufacture, issue, and use: A chain of diminishing returns? *Transfus Med Rev* 2004; **18**: 279–292.
[11] Villar A, Jimenez-Yuste V, Quintana M, Hernandez-Navarro F. The use of haemostatic drugs in haemophilia: Desmopressin and antifibrinolytic agents. *Haemophilia* 2002; **8**: 189–193.
[12] Bishton M, Chopra R. The role of granulocyte transfusions in neutropenic patients. *Br J Haematol* 2004; **127**: 501–508.
[13] Beckman N, Nicholson G, Ashford M, Hambleton R. Blood pack fault monitoring. *Vox Sang* 2004; **87**: 272–279.
[14] Goodnough LT. Autologous blood donation. *Crit Care* 2004; **8(Suppl 2)**: S49–S52.
[15] Pomper GJ, Wu Y, Snyder EL. Risks of transfusion-transmitted infections: 2003. *Curr Opin Hematol* 2003; **10**: 412–418.
[16] Pauli G. Tissue safety in view of CJD and variant CJD. *Cell Tissue Bank* 2005; **6**: 191–200.
[17] Cervenakova L, Brown P. Advances in screening test development for transmissible spongiform encephalopathies. *Expert Rev Anti Infect Ther* 2004; **2**: 873–880.
[18] Carson JL, Reynolds RC. In search of the transfusion threshold. *Hematology* 2005; **10 (Suppl 1)**: 86–88.
[19] Hardy JF. Current status of transfusion triggers for red blood cell concentrates. *Transfus Apher Sci* 2004; **31**: 55–66.
[20] Pace N. The increase in hypoxia tolerance of normal men accompanying the polycythemia induced by transfusion of erythocytes. *Am J Physiol* 1947; **148**: 152–163.
[21] Audran M, Varlet-Marie E. Blood boosting today. *Bull Acad Natl Med* 2004; **188**: 945–953.
[22] McArdle WD, Katch FI, Katch VL. Exercise Physiology. In McArdle WD, Katch FI, Katch VL, Eds. Energy, Nutrition, and Human Performance. 2nd Ed. Pp 409–411. Philadelphia: Lea and Febiger, 1986.
[23] Gledhill N. Blood doping and related issues: A brief review. *Med Sci Sports Exerc* 1982; **14**: 183–189.
[24] Ghaphery NA. Performance-enhancing drugs. *Orthop Clin North Am* 1995; **26**: 433–442.
[25] Smith DA, Perry PJ. The efficacy of ergogenic agents in athletic competition. Part II: Other performance-enhancing agents. *Ann Pharmacother* 1992; **26**: 653–659.
[26] Lippi G, Franchini M, Guidi GC. Haematological testing and antidoping policies. *Int J Sports Med* 2005; **26**: 508–509.
[27] Nelson M, Ashenden M, Langshaw M, Popp H. Detection of homologous blood transfusion by flow cytometry: A deterrent against blood doping. *Haematologica* 2002; **87**: 881–882.
[28] Nelson M, Popp H, Sharpe K, Ashenden M. Proof of homologous blood transfusion through quantification of blood group antigens. *Haematologica* 2003; **88**: 1284–1295.
[29] Drexler C, Glock B, Vadon M, Staudacher E, Dauber EM, Ulrich S, Reisacher RB, Mayr WR, Lanzer G, Wagner T. Tetragametic chimerism detected in a healthy woman with mixed-field agglutination reactions in ABO blood grouping. *Transfusion* 2005; **45**: 698–703.

[30] Malcovati L, Pascutto C, Cazzola M. Hematologic passport for athletes competing in endurance sports: A feasibility study. *Haematologica* 2003; **88**: 570–571.

[31] Lee-Huang S. Cloning and expression of human erythropoietin cDNA in Escherichia coli. *Proc Natl Acad Sci USA* 1984; **81**: 2708–2712.

[32] Franchini M, Gandini G, Lippi G, De Gironcoli M, Cantini M, Aprili G. Clinical use of erythropoietin. *Recenti Prog Med* 2004; **95**: 129–136.

[33] Cazzola M. Further concerns about the medical risks of blood doping. *Haematologica* 2002; **87**: 232.

[34] Joyner MJ. VO2MAX, blood doping, and erythropoietin. *Br J Sports Med* 2003; **37**: 190–191.

[35] Wilber RL. Detection of DNA-recombinant human epoetin-alfa as a pharmacological ergogenic aid. *Sports Med* 2002; **32**: 125–142.

[36] Carnot P, Deflandre C. Sur l'activité hémopoïétique du serum au cours de la régénération du sang. *CR Acad Sci Paris* 1906; **143**: 432–435.

[37] Krumdieck N. Erythropoietic substance in the serum of anemic animals. *Proc Soc Exp Biol Med* 1943; **54**: 14–17.

[38] Ebert BL, Bunn HF. Regulation of the erythropoietin gene. *Blood* 1999; **94**: 1864–1877.

[39] Lacombe C, Mayeux P. Biology of erythropoietin. *Haematologica* 1998; **83**: 724–732.

[40] Krantz SB. Erythropoietin. *Blood* 1991; **77**: 419–434.

[41] Choi D, Kim M, Park J. Erythropoietin: Physico- and biochemical analysis. *J Chromatog B Biomed Appl* 1996; **687**: 189–199.

[42] Bazan JF. A novel family of growth factor receptors: A common binding domain in the growth hormone, prolactin, erythropoietin and IL-6 receptors, and the p75 IL-2 receptor β chain. *Biochem Biophys Res Commun* 1989; **164**: 788–792.

[43] Wen D, Boissel JP, Tracy TE, Gruninger RH, Mulcahy LS, Czelusniak J, Goodman M, Bunn HF. Erythropoietin structure-function relationships: High degree of sequence homology among mammals. *Blood* 1993; **82**: 1507–1516.

[44] Maxwell PH, Ferguson DJ, Osmond MK, Pugh CW, Heryet A, Doe BG, Johnson MH, Ratcliffe PJ. Expression of a homologously recombined erythopoietin-SV40 T antigen fusion gene in mouse liver: Evidence for erythropoietin production by Ito cells. *Blood* 1994; **84**: 1823–1830.

[45] Maxwell PH, Osmond MK, Pugh CW, Heryet A, Nicholls LG, Tan CC, Doe BG, Ferguson DJ, Johnson MH, Ratcliffe PJ. Identification of the renal erythropoietin-producing cells using transgenic mice. *Kidney Int* 1993; **44**: 1149–1162.

[46] Schmit RM, Bruyns E, Snodgrass H. Haematopoietic development of embryonic stem cells in vitro; cytokine and receptor gene expression. *Genes Dev* 1991; **5**: 728–740.

[47] Yasuda Y, Nagao M, Okano M, Masuda S, Sasaki R, Konishi H, Tanimura T. Localization of erythropoietin and erythropoietin-receptor in postimplantation mouse embryos. *Dev Growth Differ* 1993; **35**: 711–722.

[48] Marti HH, Wenger RH, Rivas LA, Straumann U, Digicaylioglu M, Henn V, Yonekawa Y, Bauer C, Gassmann M. Erythropoietin gene expression in human, monkey and murine brain. *Eur J Neurosci* 1996; **8**: 666–676.

[49] Yasuda Y, Masuda S, Chikuma M, Inoue K, Nagao M, Sasaki R. Estrogen-dependent production of erythropoietin in uterus and its implication in uterine angiogenesis. *J Biol Chem* 1998; **273**: 25381–25387.

[50] Yasuda Y, Fujita Y, Musha T, Tanaka H, Shiokawa S, Nakamatsu K, Mori S, Matsuo T, Nakamura Y. Expression of erythropoietin in human female reproductive organs. *Ital J Anat Embryol* 2001; **106 (2 Suppl 2)**: 215–222. **Q5**

[51] Fandrey J, Bunn HF. In vivo and in vitro regulation of erythropoietin mRNA: Measurement by competitive polymerase chain reaction. *Blood* 1993; **81**: 617–623.

[52] Varlet-Marie E, Gaudard A, Audran M, Bressolle F. Pharmacokinetics/pharmacodynamics of recombinant human erythropoietins in doping control. *Sports Med* 2003; **33**: 301–315.

[53] Varlet-Marie E, Gaudard A, Audran M, Gomeni R, Bressolle F. Pharmacokinetic-pharmacodynamic modelling of recombinant human erythropoietin in athletes. *Int J Sports Med* 2003; **24**: 252–257.

[54] Parisotto R, Gore CJ, Emslie KR, Ashenden MJ, Brugnara C, Howe C, Martin DT, Trout GJ, Hahn AG. A novel method utilising markers of altered erythropoiesis for the detection of recombinant human erythropoietin abuse in athletes. *Haematologica* 2000; **85**: 564–572.

[55] Connes P, Perrey S, Varray A, Prefaut C, Caillaud C. Faster oxygen uptake kinetics at the onset of submaximal cycling exercise following 4 weeks recombinant human erythropoietin (r-HuEPO) treatment. *Pflugers Arch* 2003; **447**: 231–238.

[56] Storring PL, Tiplady RJ, Gaines Das RE, Stenning BE, Lamikanra A, Rafferty B, Lee J. Epoetin alfa and beta differ in their erythropoietin isoform compositions and biological properties. *Br J Haematol* 1998; **100**: 79–89.

[57] Deicher R. Hörl WH. Differentiating factors between erythropoiesis-stimulating agents: A guide to selection for anaemia of chronic kidney disease. *Drugs* 2004; **64**: 499–509.

[58] Elliott S, Egrie J, Browne J, Lorenzini T, Busse L, Rogers N, Ponting I. Control of rHuEPO biological activity: The role of carbohydrate. *Exp Hematol* 2004; **32**: 1146–1155.

[59] MacDougall IC. Novel erythropoiesis stimulating protein. *Semin Nephrol* 2000; **20**: 375–381.

[60] Fisher JW. Erythropoietin: Physiology and pharmacology update. Minireview. *Exp Biol Med* 2003; **228**: 1–14.

[61] Macdougall IC, Gray SJ, Elston O, Breen C, Jenkins B, Browne J, Egrie J. Pharmacokinetics of novel erythropoieis protein compared with epoetin-alfa in dialysis patients. *J Am Soc Nephrol* 1999; **10**: 2392–2395.

[62] Macdougall IC. CERA (Continuous Erythropoietin Receptor Activator): A new erythropoiesis-stimulating agent for the treatment of anemia. *Curr Hematol Rep* 2005;**4**: 436–440.

[63] Cases A. Update in nephrology. Highlights from the 36th Annual Meeting of the American Society of Nephrology. *Drugs Today (Barc)* 2004; **40**: 81–92.

[64] Lippi G, Guidi GC. Gene manipulation and improvement of athletic performances: New strategies in blood doping. *Br J Sports Med* 2004; **38**: 641.

[65] Fandrey J. Oxygen-dependent and tissue-specific regulation of erythropoietin gene expression. *Am J Physiol Regul Integr Comp Physiol* 2004; **286**: R977–R988.

[66] López-Barneo J, Pardal R, Ortega-Sáenz P. Cellular mechanisms of oxygen sensing. *Annu Rev Physiol* 2001; **63**: 259–287.

[67] López-Barneo J. Oxygen-sensitive ion channels: How ubiquitous are they? *Trends Neurosci* 1994; **17**: 133–135.

[68] Buckler KJ. A novel oxygen-sensitive potassium current in rat carotid body type I cells. *J Physiol* 1997; **498**: 649–662.

[69] Rychkov GY, Adams MB, McMillen IC, Roberts ML. Oxygen-sensing mechanisms are present in the chromaffin cells of the sheep adrenal medulla before birth. *J Physiol* 1998; **509**: 887–893.

[70] Youngson C, Nurse C, Yeger H, Cutz E. Oxygen sensing in airway chemoreceptors. *Nature* 1993; **365**: 153–155.

[71] López-Barneo J. Oxygen and glucose sensing by carotid body glomus cells. *Curr Opin Neurobiol* 2003; **13**: 493–499.

[72] Semenza GL, Wang GL. A nuclear factor induced by hypoxia via de novo protein synthesis bind to the human erythropoietin gene enhancer at a site required for transcriptional activation. *Mol Cell Biol* 1992; **12**: 5447–5454.

[73] Wang GL, Semenza GL. Purification and characterization of hypoxia-inducible factor 1. *J Biol Chem* 1995; **270**: 1230–1237.

[74] Marx J. How cells endure low oxygen. *Science* 2004; **303**: 1454–1456.

[75] Vanrenterghem Y, Vanwalleghem J. Benefits and concerns of treating pre-dialysis and renal transplant patients with recombinant human erythropoietin. *Nephrol Dial Transplant* 1998; **13 (Suppl 2)**: 13–15.

[76] Lage JM, Panizo C, Masdeu J, Rocha E. Cyclist's doping associated with cerebral sinus thrombosis. *Neurology* 2002; **58**: 665.

[77] Rosenzweig MQ, Bender CM, Lucke JP, Yasko JM, Brufsky AM. The decision to prematurely terminate a trial of R-HuEPO due to thrombotic events. *J Pain Symptom Manage* 2004; **27**: 185–190.

[78] Delanty N, Vaughan C, Frucht S, Stubgen P. Erythropoietin-associated hypertensive posterior leukoencephalopathy. *Neurology* 1997; **49**: 686–689.

[79] Beccari M. Seizures in dialysis patients treated with recombinant erythropoietin. Review of the literature and guidelines for prevention. *Int J Artif Organs* 1994; **17**: 5–13.

[80] Goodnough LT, Price TH, Friedman KD, Johnston M, Ciavarella D, Khan N, Sacher R, Vogler WR, Wissel M, Abels RI. A phase III trial of recombinant human erythropoietin therapy in nonanemic orthopedic patients subjected to aggressive removal of blood for autologous use: Dose, response, toxicity, and efficacy. *Transfusion* 1994; **34**: 66–71.

Q6

[81] Bennett CL, Luminari S, Nissenson AR, Tallman MS, Klinge SA, McWilliams N, McKoy JM, Kim B, Lyons EA, Trifilio SM, Raisch DW, Evens AM, Kuzel TM, Schumock GT, Belknap SM, Locatelli F, Rossert J, Casadevall N. Pure red-cell aplasia and epoetin therapy. *N Engl J Med* 2004; **351**: 1403–1408.

[82] Lim LC. Acquired red cell aplasia in association with the use of recombinant erythropoietin in chronic renal failure. *Hematology* 2005; **10**: 255–259.

[83] Casadevall N, Cournoyer D, Marsh J, Messner H, Pallister C, Parker-Williams J, Rossert J. Recommendations on haematological criteria for the diagnosis of epoetin-induced pure red cell aplasia. *Eur J Haematol* 2004; **73**: 389–396.

[84] Piron M, Loo M, Gothot A, Tassin F, Fillet G, Beguin Y. Cessation of intensive treatment with recombinant human erythropoietin is followed by secondary anemia. *Blood* 2001; **97**: 442–448.

[85] Juul SE, Yachnis AT, Christensen RD. Tissue distribution of erythropoietin and erythropoietin receptor in the developing human fetus. *Early Hum Dev* 1998; **52**: 235–249.

[86] Yasuda Y, Fujita Y, Matsuo T, Koinuma S, Hara S, Tazaki A, Onozaki M, Hashimoto M, Musha T, Ogawa K, Fujita H, Nakamura Y, Shiozaki H, Utsumi H. Erythropoietin regulates tumour growth of human malignancies. *Carcinogenesis* 2003; **24**: 1021–1029.

[87] Broudy VC, Lin N, Brice M, Nakamoto B, Papayannopoulou T. Erythropoietin receptor characteristics on primary human erythroid cells. *Blood* 1991; **77**: 2583–2590.

[88] Lippi G, Guidi G. Laboratory screening for erythropoietin abuse in sport: An emerging challenge. *Clin Chem Lab Med* 2000; **38:** 13–19.

[89] Gareau R, Audran M, Baynes RD, Flowers CH, Duvallet A, Senecal L, Brisson GR. Erythropoietin abuse in athletes. *Nature* 1996; **380**: 113.

[90] Lippi G, Manzato F, Franchini M, Guidi G. Relationship between hematocrit, primary hemostasis, and cardiovascular disease in athletes. *Am Heart J* 2002; **144**: E14.

[91] Kazlauskas R, Howe C, Trout G. Strategies for rhEPO detection in sport. *Clin J Sport Med* 2002; **12**: 229–235.

[92] Sharpe K, Hopkins W, Emslie KR, Howe C, Trout GJ, Kazlauskas R, Ashenden MJ, Gore CJ, Parisotto R, Hahn AG. Development of reference ranges in elite athletes for markers of altered erythropoiesis. *Haematologica* 2002; **87**: 1248–1257.

[93] Lippi G, Franchini M, Guidi G. Second generation blood tests to detect erythropoietin abuse by athletes: Effective but not preventive? *Haematologica* 2004; **89**: ELT05. **Q7**

[94] Robinson N, Saugy M, Mangin P. Effects of exercise on the secondary blood markers commonly used to suspect erythropoietin doping. *Clin Lab* 2003; **49**: 57–62.

[95] Ashenden MJ, Gore CJ, Parisotto R, Sharpe K, Hopkins WG, Hahn AG. Effect of altitude on second-generation blood tests to detect erythropoietin abuse by athletes. *Haematologica* 2003; **88**: 1053–1062.

[96] Lippi G, Salvagno GL, Brocco G, Guidi GC. Preanalytical variability in laboratory testing: Influence of the blood drawing technique. *Clin Chem Lab Med* 2005; **43**: 319–325.

[97] Lippi G, Salvagno GL, Solero GP, Franchini M, Guidi GC. Stability of blood cell counts, hematologic parameters and reticulocytes indexes on the Advia A120 hematologic analyzer. *J Lab Clin Med* 2005; **146**: 333–340.

[98] Villarrubia J. Decision rules for hematology review. An international perspective. *Lab Hematol* 2004; **10**: 158–159.

[99] Breidbach A, Catlin DH, Green GA, Tregub I, Truong H, Gorzek J. Detection of recombinant human erythropoietin in urine by isoelectric focusing. *Clin Chem* 2003; **49**: 901–907.

[100] Wide L, Bengtsson C, Berglund B, Ekblom B. Detection in blood and urine of recombinant erythropoietin administered to healthy men. *Med Sci Sports Exerc* 1995; **27**: 1569–1576.

[101] Catlin DH, Breidbach A, Elliott S, Glaspy J. Comparison of isoelectric focusing patterns of darbepoetin alfa, recombinant human erythropoietin, and endogenous erythropoietin from human urine. *Clin Chem* 2002; **48**: 2057–2059.

[102] Lasne F, Martin L, Crepin N, de Ceaurriz J. Detection of isoelectric profiles of erythropoietin in urine: Differentiation of natural and administered recombinant hormones. *Anal Biochem* 2002; **311**: 119–126.

[103] Lopez-Soto-Yarritu P, Diez-Masa JC, Cifuentes A, de Frutos M. Improved capillary isoelectric focusing method for recombinant erythropoietin analysis. *J Chromatogr A* 2002; **968**: 221–228.

[104] Varlet-Marie E, Audran M, Lejeune M, Bonafoux B, Sicart MT, Marti J, Piquemal D, Commes T. Analysis of human reticulocyte genes reveals altered erythropoiesis: Potential use to detect recombinant human erythropoietin doping. *Haematologica* 2004; **89**: 991–997.

[105] Brugnara C. Reticulocyte cellular indices: A new approach in the diagnosis of anemias and monitoring of erythropoietic function. *Crit Rev Clin Lab Sci* 2000; **37**: 93–130.

[106] Wide L, Wikstrom B, Eriksson K. A new principle suggested for detection of darbepoetin-alpha (NESP) doping. *Ups J Med Sci* 2003; **108**: 229–238.

[107] Rusko HK, Tikkanen HO, Peltonen JE. Altitude and endurance training. *J Sports Sci* 2004; **22**: 928–944.

[108] Wu T, Li S, Ward MP. Tibetans at extreme altitude. *Wilderness Environ Med* 2005; **16**: 47–54.

[109] Ge RL, Witkowski S, Zhang Y, Alfrey C, Sivieri M, Karlsen T, Resaland GK, Harber M, Stray-Gundersen J, Levine BD. Determinants of erythropoietin release in response to short-term hypobaric hypoxia. *J Appl Physiol* 2002; **92**: 2361–2367.

[110] Gore CJ, Hahn AG, Burge CM, Telford RD. VO2max and haemoglobin mass of trained athletes during high intensity training. *Int J Sports Med* 1997; **18:** 477–482.

[111] Coote JH. Medicine and mechanisms in altitude sickness: Recommendations. *Sports Medicine* 1995; **20**: 148–159.

[112] Fandrey J. High-altitude polycythemia. *Haematologica* 2005; **90**: 1.

[113] Leon-Velarde F, Maggiorini M, Reeves JT, Aldashev A, Asmus I, Bernardi L, Ge RL, Hackett P, Kobayashi T, Moore LG, Penaloza D, Richalet JP, Roach R, Wu T, Vargas E, Zubieta-Castillo G, Zubieta-Calleja G. Consensus statement on chronic and subacute high altitude diseases. *High Alt Med Biol* 2005; **6**: 147–157.

[114] Chapman RF, Stray-Gundersen J, Levine BD. Individual variation in response to altitude training. *J Appl Physiol* 1998; **85**: 1448–1456.

[115] Alfrey CP, Rice L, Udden MM, Driscoll TB. Neocytolysis: Physiological down-regulator of red-cell mass. *Lancet* 1997; **349**: 1389–1390.

[116] Rice L, Alfrey CP. The negative regulation of red cell mass by neocytolysis: Physiologic and pathophysiologic manifestations. *Cell Physiol Biochem* 2005; **15**: 245–250.

[117] Rice L, Ruiz W, Driscoll T, Whitley CE, Tapia R, Hachey DL, Gonzales GF, Alfrey CP. Neocytolysis on descent from altitude: A newly recognized mechanism for the control of red cell mass. *Ann Intern Med* 2001; **134**: 652–626.

[118] Harris BA Jr, Epstein PE. Out of thin air: The evolving enigma of erythropoietin and neocytolysis. *Ann Intern Med* 2001; **134**: 710–712.

[119] Rice L, Alfrey CP. Down-regulation of red cell mass by neocytolysis in space and on Earth. *Eur J Physiol* 2000; **441**: R91–R94.

[120] Trial J, Rice L. Erythropoietin withdrawal leads to the destruction of young red cells at the endothelial-macrophage interface. *Curr Pharm Des* 2004; **10**: 183–190.

[121] Testa U. Apoptotic mechanisms in the control of erythropoiesis. *Leukemia* 2004; **18**: 1176–1199.

[122] Dick FW. Training at altitude in practice. *Int J Sports Med* 1992; **13**: S203–S206.

[123] Hahn AG, Gore CJ. The effect of altitude on cycling performance: A challenge to traditional concepts. *Sports Med* 2001; **31:** 533–557.

[124] Mairbaurl H, Schobersberger W, Humpeler E, Hasibeder E, Fischer W, Raas E. Beneficial effects of exercising at moderate altitude on oxygen transport and on exercise performance. *Pflügers Arch* 1986; **406**: 594–599.

[125] Bigard AX, Brunet A, Guezennec CY, Monod H. Skeletal muscle changes after endurance training at high altitude. *J Appl Physiol* 1991; **71**: 2114–2121.

[126] Eckardt K, Boutellier U, Kurtz A, Schopen M, Koller E, Bauer C. Rate of erythropoietin formation in humans in response to acute hypobaric hypoxia. *J Appl Physiol* 1989; **66**: 1785–1788.

[127] Levine BD, Stray-Gundersen J. "Living high-training low": Effect of moderate-altitude acclimatization with low-altitude training on performance. *J Appl Physiol* 1997; **83**: 102–112.

[128] Morton JP, Cable NT. The effects of intermittent hypoxic training on aerobic and anaerobic performance. *Ergonomics* 2005; **48**: 1535–1546.

[129] Levine BD, Stray-Gundersen J. A practical approach to altitude training: Where to live and train for optimal performance enhancement. *Int J Sports Med* 1992; **13**: S209–S212.

[130] Stray-Gundersen J, Chapman RF, Levine BD. "Living high-training low" altitude training improves sea level performance in male and female elite runners. J*Appl Physiol* 2001; **91**: 1113–1120.

[131] Scoggin CH, Doekel RD, Kruger MH, Zwillich CW, Weil JV. Familial aspects of decreased hypoxic drive in endurance athletes. *J Appl Physiol* 1978; **44**: 464–468.

[132] Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension. *Proc Natl Acad Sci USA* 1995; **92**: 5510–5514.

[133] Murray TH. An Olympic tail? *Nat Rev Genet* 2003; **4**: 494.

[134] Lippi G, Solero GP, Guidi G. Athletes genotyping: Ethical and legal issues. *Int J Sports Med* 2004; **25**: 159.

[135] Schmidt W. Effects of intermittent exposure to high altitude on blood volume and erythropoietic activity. *High Alt Med Biol* 2002; **3**: 167–176.

[136] Julian CG, Gore CJ, Wilber RL, Daniels JT, Fredericson M, Stray-Gundersen J, Hahn AG, Parisotto R, Levine BD. Intermittent normobaric hypoxia does not alter performance or erythropoietic markers in highly trained distance runners. *J Appl Physiol* 2004; **96**: 1800–1807.

[137] Rodríguez FA. Intermittent hypoxia: An alternative to acclimatization to high altitude and enhancement of athletic performance? *Am J Med Sports* 2002; **4**: 385–391.

[138] Rodriguez FA, Ventura JL, Casas M, Casas H, Pages T, Rama R, Ricart A, Palacios L, Viscor G. Erythropoietin acute reaction and haematological adaptations to short, intermittent hypobaric hypoxia. *Eur J Appl Physiol* 2000; **82**: 170–177.

[139] Abellán R, Remacha AF, Ventura R, Sardà MP, Segura J, Rodríguez FA. Hematologic response to four weeks of intermittent hypobaric hypoxia in highly trained athletes. *Haematologica* 2005; **90**: 126–127.

[140] Roels B, Millet GP, Marcoux CJ, Coste O, Bentley DJ, Candau RB. Effects of hypoxic interval training on cycling performance. *Med Sci Sports Exerc* 2005; **37**: 138–146.

[141] Lundby C, Nielsen TK, Dela F, Damsgaard R. The influence of intermittent altitude exposure to 4100 m on exercise capacity and blood variables. *Scand J Med Sci Sports* 2005; **15**: 182–187.

[142] Serebrovskaya TV. Intermittent hypoxia research in the former Soviet Union and the Commonwealth of Independent States: History and review of the concept and selected applications. *High Alt Med Biol* 2002: 3: 205–221.

[143] Levine BD. Intermittent hypoxic training: Fact and fancy. *High Alt Med Biol* 2002; **3**: 177–193.

[144] Wilber RL. Current trends in altitude training. *Sports Med* 2001; **31**: 249–265.

[145] Koistinen PO, Rusko H, Irjala K, Rajamaki A, Penttinen K, Sarparanta VP, Karpakka J, Leppaluoto J. EPO, red cells, and serum transferrin receptor in continuous and intermittent hypoxia. *Med Sci Sports Exerc* 2000; **32**: 800–804.

[146] Benoit H, Germain M, Barthelemy JC, Denis C, Castells J, Dormois D, Lacour JR, Geyssant A. Pre-acclimatization to high altitude using exercise with normobaric hypoxic gas mixtures. *Int J Sports Med* 1992; **13**: S213–S216. **Q11**

[147] Bernardi L. Interval hypoxic training. *Adv Exp Med Biol* 2001; **502**: 377–399.

[148] Ashenden MJ, Hahn AG, Martin DT, Logan P, Parisotto R, Gore CJ. A comparison of the physiological response to simulated altitude exposure and r-HuEpo administration. *J Sports Sci* 2001; **19**: 831–837.

[149] Bahrke MS, Shukitt-Hale B. Effects of altitude on mood, behaviour and cognitive functioning: A review. *Sports Med* 1993; **16**: 97–125.

[150] American College of Physicians. Practice strategies for elective red blood cell transfusions. *Ann Intern Med* 1992; **116**: 403–406.

[151] Scott MG, Kucik DF, Goodnough LT, Monk TG. Blood substitutes: Evolution and future applications. *Clin Chem* 1997; **43**: 1724–1731.

[152] Ma Z, Monk TG, Goodnough LT, McClellan A, Gawryl M, Clark T, Moreira P, Keipert PE, Scott MG. Effect of hemoglobin- and Perflubron-based oxygen carriers on common clinical laboratory tests. *Clin Chem* 1997; **43**: 1732–1737.

[153] Goodnough LT, Scott MG, Monk TG. Oxygen carriers as blood substitutes. Past, present, and future. *Clin Orthop Relat Res* 1998; **357**: 89–100.

[154] Stowell CP. What happened to blood substitutes? *Transfus Clin Biol* 2005; **12**: 374–379.

[155] Klein HG. Blood substitutes: How close to a solution? *Dev Biol (Basel)* 2005; **120**: 45–52.

[156] Keipert PE. Perfluorochemical emulsions: Future alternatives to transfusion. *Blood Subst Princ Meth Prod Clin Trials* 1998; **2**: 127–156.

[157] Wolfson MR, Shaffer TH. Pulmonary applications of perfluorochemical liquids: Ventilation and beyond. *Paediatr Respir Rev* 2005; **6**: 117–127.

[158] Spahn DR. Blood substitutes. Artificial oxygen carriers: Perfluorocarbon emulsions. *Crit Care* 1999; **3**: R93–R97.
[159] Riess JG. Understanding the fundamentals of perfluorocarbons and perfluorocarbon emulsions relevant to in vivo oxygen delivery. *Artif Cells Blood Substit Immobil Biotechnol* 2005; **33**: 47–63.
[160] Speit G, Dennog C, Radermacher P, Rothfuss A. Genotoxicity of hyperbaric oxygen. *Mutat Res* 2002; **512**: 111–119.
[161] Patel S, Mehra A. Modeling of oxygen transport in blood-perfluorocarbon emulsion mixtures: Part II: Tissue oxygenation. *ASAIO J* 1998; **44**: 157–165.
[162] Spence RK. Perfluorocarbons in the twenty first century: Clinical applications as transfusion alternatives. *Artif Cells Blood Substit Immobil Biotechnol* 1995; **23**: 367–380.
[163] Clark LC, Gollan F. Survival of mammals breathing liquids equilibrated with oxygen at atmospheric pressure. *Science* 1966; **152**: 1755–1756.
Q12 [164] Hill SE. Oxygen therapeutics. Current concepts. *Can J Anaesth* 2001; **48**: S32–S40.
[165] Flaim SF, Hazard DR, Hogan J, Peters RM. Characterization and mechanism of side-effects of Oxygent HT (highly concentrated fluorocarbon emulsion) in swine. *Artif Cells Blood Substit Immobil Biotechnol* 1994; **22**: 1511–1515.
[166] Noveck RJ, Shannon EJ, Leese PT, Shorr JS, Flaim KE, Keipert PE, Woods CM. Randomized safety studies of intravenous perflubron emulsion. II. Effects on immune function in healthy volunteers. *Anesth Analg* 2000; **91**: 812–822.
[167] Fernandez R, Sarma V, Younkin E, Hirschl RB, Ward PA, Younger JG. Exposure to perflubron is associated with decreased Syk phosphorylation in human neutrophils. *J Appl Physiol* 2001; **91**: 1941–1947.
[168] Smith DJ, Lane TA. Effect of high concentration perflubron emulsion on platelet function. *Biomater Artif Cells Immobilization Biotechnol* 1993; **21**: 173–181.
[169] Merz U, Klosterhalfen B, Kellinghaus M, Peschgens T, Pluschke S, Hoernchen H. Effects of single and multiple doses of perfluorocarbon in comparison with continuous partial liquid ventilation on gas exchange and lung pathology in newborn surfactant-depleted pigs. *Crit Care Med* 2001; **29**: 645–651.
[170] Audran M, Krafft MP, De Ceaurriz J, Maturin JC, Sicart MT, Marion B, Bougard G, Bressolle F. Determination of perfluorodecalin and perfluoro-N-methylcyclohexylpiperidine in rat blood by gas chromatography-mass spectrometry. *J Chromatogr B Biomed Sci Appl* 2000; **745**: 333–343.
[171] Shaffer TH, Foust R 3rd, Wolfson MR, Miller TF Jr. Analysis of perfluorochemical elimination from the respiratory system. *J Appl Physiol* 1997; **83**: 1033–1040.
[172] Mazzoni M, Nugent L, Klein D, Hoffman J, Sekins KM, Flaim SF. Dose monitoring in partial liquid ventilation by infrared measurement of expired perfluorochemicals. *Biomed Instrum Technol* 1999; **33**: 356–364.
[173] Proquitte H, Rudiger M, Wauer RR, Schmalisch G. Breathing gas perfluorocarbon measurements using an absorber filled with zeolites. *Br J Anaesth* 2003; **91**: 736–738.
[174] Schumacher YO, Ashenden M. Doping with artificial oxygen carriers: An update. *Sports Med* 2004; **34**: 141–150.
[175] Spahn DR, Kocian R. Artificial O2 carriers: Status in 2005. *Curr Pharm Des* 2005; **11**: 4099–4114.
[176] Chang TMS. Oxygen carriers. *Curr Opin Investig Drugs* 2002; **3**: 1187–1190.
[177] Eike JH, Palmer AF. Oxidized mono-, di-, tri-, and polysaccharides as potential hemoglobin cross-linking reagents for the synthesis of high oxygen affinity artificial blood substitutes. *Biotechnol Prog* 2004; **20**: 953–962.
[178] Bobofchak KM, Mito T, Texel SJ, Bellelli A, Nemoto M, Traystman RJ, Koehler RC, Brinigar WS, Fronticelli C. A recombinant polymeric hemoglobin with conformational, functional, and physiological characteristics of an in vivo O2 transporter. *Am J Physiol Heart Circ Physiol* 2003; **285**: H549–H561.
[179] Tsai CH, Ho C. Recombinant hemoglobins with low oxygen affinity and high cooperativity. *Biophys Chem* 2002; **98**: 15–25.
[180] Lieberthal W, Fuhro R, Freedman JE, Toolan G, Loscalzo J, Valeri CR. O-raffinose cross-linking markedly reduces systemic and renal vasoconstrictor effects of unmodified human hemoglobin. *J Pharmacol Exp Ther* 1999; **288**: 1278–1287.
[181] Talarico TL, Guise KJ, Stacey CJ. Chemical characterization of pyridoxalated hemoglobin polyoxyethylene conjugate. *Biochim Biophys Acta* 2000; **1476**: 53–65.

[182] Kim HW, Greenburg AG. Artificial oxygen carriers as red blood cell substitutes: A selected review and current status. *Artif Organs* 2004; 28: 813–828.

[183] Moore EE, Johnson JL, Cheng AM, Masuno T, Banerjee A. Insights from studies of blood substitutes in trauma. *Shock* 2005; **24**: 197–205.

[184] Hughes GS Jr, Francome SF, Antal EJ, Adams WJ, Locker PK, Yancey EP, Jacobs EE Jr. Hematologic effects of a novel hemoglobin-based oxygen carrier in normal male and female subjects. *J Lab Clin Med* 1995; **126**: 444–451.

[185] Hughes GS Jr, Antal EJ, Locker PK, Francom SF, Adams WJ, Jacobs EE Jr. Physiology and pharmacokinetics of a novel hemoglobin-based oxygen carrier in humans. *Crit Care Med* 1996; **24**: 756–764.

[186] Hughes GS Jr, Yancey EP, Albrecht R, Locker PK, Francom SF, Orringer EP, Antal EJ, Jacobs EE Jr. Hemoglobin-based oxygen carrier preserves submaximal exercise capacity in humans. *Clin Pharmacol Ther* 1995; **58**: 434–443.

[187] Resta TC, Walker BR, Eichinger MR, Doyle MP. Rate of NO scavenging alters effects of recombinant hemoglobin solutions on pulmonary vasoreactivity. *J Appl Physiol* 2002; **93**: 1327–1336.

[188] Scatena R, Giardina B. O-raffinose-polymerised haemoglobin. A biochemical and pharmacological profile of an oxygen carrier. *Expert Opin Biol Ther* 2001; **1**: 121–127.

[189] Goebel C, Alma C, Howe C, Kazlauskas R, Trout G. Methodologies for detection of hemoglobin-based oxygen carriers. *J Chromatogr Sci* 2005; **43**: 39–46.

[190] Lasne F, Crepin N, Ashenden M, Audran M, de Ceaurriz J. Detection of hemoglobin-based oxygen carriers in human serum for doping analysis: Screening by electrophoresis. *Clin Chem* 2004; **50**: 410–415.

[191] Guan F, Uboh CE, Soma LR, Luo Y, Jahr JS, Driessen B. Confirmation and quantification of hemoglobin-based oxygen carriers in equine and human plasma by hyphenated liquid chromatography tandem mass spectrometry. *Anal Chem* 2004; **76**: 5127–5135.

[192] Varlet-Marie E, Ashenden M, Lasne F, Sicart MT, Marion B, de Ceaurriz J, Audran M. Detection of hemoglobin-based oxygen carriers in human serum for doping analysis: Confirmation by size-exclusion HPLC. *Clin Chem* 2004; **50**: 723–731.

[193] Kenakin T. Allosteric modulators: The new generation of receptor antagonist. *Mol Interv* 2004; **4**: 222–229.

[194] Randad RS, Mahran MA, Mehanna AS, Abraham DJ. Allosteric modifiers of hemoglobin, I: Design, synthesis, testing, and structure-allosteric activity relationship of novel hemoglobin oxygen affinity decreasing agents. *J Med Chem* 1991; **34**: 752–757.

[195] Papassotiriou I, Kister J, Griffon N, Stamoulakatou A, Abraham DJ, Marden MC, Loukopoulos D, Poyart C. Modulating the oxygen affinity of human fetal haemoglobin with synthetic allosteric modulators. *Br J Haematol* 1998; **102**: 1165–1167.

[196] Abraham DJ, Wireko FC, Randad RS, Poyart C, Kister J, Bohn B, Liard JF, Kunert MP. Allosteric modifiers of hemoglobin: 2-[4-[[(3,5-disubstituted anilino)carboxy]methyl]phenoxy]-2-methylpropionic acid derivatives that lower the oxygen affinity of hemoglobin in red cell suspensions, in whole blood, and in vivo in rats. *Biochemistry* 1992; **31**: 9141–9149.

[197] Wahr JA, Gerber M, Venitz J, Baliga N. Allosteric modification of oxygen delivery by hemoglobin. *Anesth Analg* 2001; **92**: 615–620.

[198] Steffen RP, Liard JF, Gerber MJ, Hoffman SJ. Allosteric modification of hemoglobin by RSR13 as a therapeutic strategy. *Adv Exp Med Biol* 2003; **530**: 249–259.

[199] Hou H, Khan N, O'Hara JA, Grinberg OY, Dunn JF, Abajian MA, Wilmot CM, Demidenko E, Lu S, Steffen RP, Swartz HM. Increased oxygenation of intracranial tumors by efaproxyn (efaproxiral), an allosteric hemoglobin modifier: In vivo EPR oximetry study. *Int J Radiat Oncol Biol Phys* 2005; **61**: 1503–1509.

[200] Youssef AM, Safo MK, Danso-Danquah R, Joshi GS, Kister J, Marden MC, Abraham DJ. Synthesis and X-ray studies of chiral allosteric modifiers of hemoglobin. *J Med Chem* 2002; **45**: 1184–1195.

[201] Lippi G, Franchini M. The new frontiers of blood doping. *Recenti Prog Med* 2002; **93**: 1–8.

[202] [No authors listed]. Efaproxiral: GSJ 61, JP 4, KDD 86, RS 4, RSR 13. *Drugs R D* 2005; **6**: 178–185.

[203] Paranka N. Hemoglobin modifiers: Is RSR13 the next aerobic enhancer? *Bloodline Rev* 2001; **1**: 23–24.

[204] Breidbach A, Catlin DH. RSR13, a potential athletic performance enhancement agent: Detection in urine by gas chromatography/mass spectrometry. *Rapid Commun Mass Spectrom* 2001; **15**: 2379–2382.

[205] The world antidoping code. The 2005 Prohibited List International Standard. World Anti-Doping Agency 2005. http://www.wada-ama.org/rtecontent/document/list_2005.pdf. Last accessed: December 29, 2005.
[206] Heeney MM, Andrews NC. Iron homeostasis and inherited iron overload disorders: An overview. *Hematol Oncol Clin North Am* 2004; **18**: 1379–1403.
Q13 [207] Beard J, Tobin B. Iron status and exercise. *Am J Clin Nutr* 2000; **72**: 594S–597S.
[208] Brugnara C, Colella GM, Cremins J, Langley RC Jr, Schneider TJ, Rutherford CJ, Goldberg MA. Effects of subcutaneous recombinant human erythropoietin in normal subjects: Development of decreased reticulocyte hemoglobin content and iron deficient erythropoiesis. *J Lab Clin Med* 1994; **123**: 660–667.
[209] Institute of Medicine. Food and Nutrition Board. Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium and Zinc. Washington, DC: National Academy Press, 2001. (Available at http://darwin.nap.edu/books/0309072794/html/290.html)
[210] Fallon KE. Utility of hematological and iron-related screening in elite athletes. *Clin J Sport Med* 2004; **14**: 145–152.
[211] Fogelholm M. Inadequate iron status in athletes: An exaggerated problem? In Kies CV, Driskell JA, Eds. Sports Nutrition: Minerals and Electrolytes. Pp 81–95. Boca Raton: CRC Press, 1995.
[212] Nielsen P, Nachtigall D. Iron supplementation in athletes. Current recommendations. *Sports Med* 1998; **26**: 207–216.
[213] Deugnier Y, Loreal O, Carre F, Duvallet A, Zoulim F, Vinel JP, Paris JC, Blaison D, Moirand R, Turlin B, Gandon Y, David V, Megret A, Guinot M. Increased body iron stores in elite road cyclists. *Med Sci Sports Exerc* 2002; **34**: 876–880.
[214] Zotter H, Robinson N, Zorzoli M, Schattenberg L, Saugy M, Mangin P. Abnormally high serum ferritin levels among professional road cyclists. *Br J Sports Med* 2004; **38**: 704–708.
[215] Zoller H, Vogel W. Iron supplementation in athletes—first do no harm. *Nutrition* 2004; **20**: 615–619.
[216] Lippi G, Schena F, Franchini M, Salvagno GL, Guidi GC. Serum ferritin as a marker of potential biochemical iron overload in athletes. *Clin J Sport Med* 2005; **15**: 356–358.
[217] Besarab A, Frinak S, Yee J. An indistinct balance: The safety and efficacy of parenteral iron therapy. *J Am Soc Nephrol* 1999; **10**: 2029–2043.
[218] Jehn M, Clark JM, Guallar E. Serum ferritin and risk of the metabolic syndrome in U.S. adults. *Diabetes Care* 2004; **27**: 2422–2428.
[219] Afzali B, Goldsmith DJ. Intravenous iron therapy in renal failure: Friend and foe? *J Nephrol* 2004; **17**: 487–495.
[220] Chicharro JL, Hoyos J, Gomez-Gallego F, Villa JG, Bandres F, Celaya P, Jimenez F, Alonso JM, Cordova A, Lucia A. Mutations in the hereditary haemochromatosis gene HFE in professional endurance athletes. *Br J Sports Med* 2004; **38**: 418–421.
[221] Beutler E, Hoffbrand AV, Cook JD. Iron deficiency and overload. *Hematology (Am Soc Hematol Educ Program)* 2003: 40–61.
[222] Xi L, Tahler M, Yin C, Salloum F, Kukreja RC. Cobalt chloride induces delayed cardiac preconditioning in mice trough selective activation of HIF-1α and AP-1 and iNOS signaling. *Am J Physiol Heart Circ Physiol* 2004; **287**: H2369–H2375.
[223] Dery MA, Michaud MD, Richard DE. Hypoxia-inducible factor-1: Regulation by hypoxic and non-hypoxic activators. *Int J Biochem Cell Biol* 2005; **37**: 535–540.
[224] Holly RG. Studies on iron and cobalt metabolism. *JAMA* 1955; **158**: 1349–1352.
[225] Lippi G, Franchini M, Guidi GC. Cobalt chloride administration in athletes: A new perspective in blood doping? *Br J Sports Med* 2005; **39**: 872–873.
[226] Ayala-Fierro F, Firriolo JM, Carter DE. Disposition, toxicity, and intestinal absorption of cobaltous chloride in male Fischer 344 rats. *J Toxicol Environ Health A* 1999; **56**: 571–591.
[227] Pourahmad J, O'Brien PJ, Jokar F, Daraei B. Carcinogenic metal induced sites of reactive oxygen species formation in hepatocytes. *Toxicol In Vitro* 2003; **17**: 803–810.
[228] Barceloux DG. Cobalt. *Toxicol Clin Toxicol* 1999; **37**: 201–216.
[229] Lippi G, Guidi G. New scenarios in antidoping research. *Clin Chem* 2003; **49**: 2106–2107.
[230] Lippi G, Guidi GC. Gene manipulation and improvement of athletic performances: New strategies in blood doping. *Br J Sports Med* 2004; **38**: 641.
[231] Unal M, Ozer Unal D. Gene doping in sports. *Sports Med* 2004; **34**: 357–362.

[232] Pincock S. Feature: Gene doping. *Lancet* 2005; **366 (Suppl 1)**: S18–S19.
[233] Azzazy HM, Mansour MM, Christenson RH. Doping in the recombinant era: Strategies and counterstrategies. *Clin Biochem* 2005; **38**: 959–965.
[234] Bohl D, Bosch A, Cardona A, Salvetti A, Heard JM. Improvement of erythropoiesis in beta-thalassemic mice by continuous erythropoietin delivery from muscle. *Blood* 2000; **95**: 2793–2798.
[235] Gao G, Lebherz C, Weiner DJ, Grant R, Calcedo R, McCullough B, Bagg A, Zhang Y, Wilson JM. Erythropoietin gene therapy leads to autoimmune anemia in macaques. *Blood* 2004; **103**: 3300–3302.
[236] Walker MC, Mandell TC, Crawford PC, Simon GG, Cahill KS, Fernandes PJ, MacLeod JN, Byrne BJ, Levy JK. Expression of erythropoietin in cats treated with a recombinant adeno-associated viral vector. *Am J Vet Res* 2005; **66**: 450–456.
[237] Rivera VM, Gao GP, Grant RL, Schnell MA, Zoltick PW, Rozamus LW, Clackson T, Wilson JM. Long-term pharmacologically regulated expression of erythropoietin in primates following AAV-mediated gene transfer. *Blood* 2005; **105**: 1424–1230. **Q14**
[238] Lippin Y, Dranitzki-Elhalel M, Brill-Almon E. Human erythropoietin gene therapy for patients with chronic renal failure. *Blood* 2005; **106**: 2280–2286.
[239] Williams DA, Baum C. Gene therapy—New challenges ahead. *Science* 2003; **302**: 400–401.
[240] Kimmelman J. Recent developments in gene transfer: Risk and ethics. *BMJ* 2005; **330**: 79–82.
[241] Verhoeyen E, Cosset FL. Inadvertent autoimmunity in EPO gene transfer. *Blood* 2004; **103**: 3248–3249.
[242] Emanuel EJ, Wendler D, Grady C. What makes clinical research ethical? *JAMA* 2000; **283**: 2701–2711.
[243] Diamanti-Kandarakis E, Konstantinopoulos PA, Papailiou J, Kandarakis SA, Andreopoulos A, Sykiotis GP. Erythropoietin abuse and erythropoietin gene doping: Detection strategies in the genomic era. *Sports Med* 2005; **35**: 831–840.
[244] Rieu M. The fight against doping: Today and tomorrow. *Bull Acad Natl Med* 2004; **188**: 955–971.
[245] Schmidt M, Zickler P, Hoffmann G, Haas S, Wissler M, Muessig A, Tisdale JF, Kuramoto K, Andrews RG, Wu T, Kiem HP, Dunbar CE, von Kalle C. Polyclonal long-term repopulating stem cell clones in a primate model. *Blood* 2002; **100**: 2737–2743.