e-ISSN 1734-2260 from 2005

OFFICIAL JOURNAL OF POLISH SOCIETY OF SPORTS MEDICINE

ISSN 1429-0022 from 1997 till 2004
Indexed in: SportDiscus; EMBASE/Excerpta Medica; Index Copernicus

Medicina Sportiva 2007 vol. 11 Issue 2

Medicina Sportiva, Vol. 11 (2): 32-42, 2007
DOI: 10.2478/v10036-007-0007-1

NOVEL ERYTHROPOIETIC AGENTS: A THREAT TO SPORTSMANSHIP

Wolfgang Jelkmann

Institute of Physiology, University, D-23538 Luebeck, Germany

Abstract

Jelkmann W. Novel erythropoietic agents: a threat to sportsmanship. *Med Sport* 2007; 11(2): 32-42.

The mass of hemoglobin (Hb) is an important determinant of aerobic power. Red blood cell production is stimulated by the glycoprotein erythropoietin (EPO). Recombinant human EPO (rHuEPO) engineered in Chinese hamster ovary (CHO) cell cultures (Epoetin alfa and Epoetin beta) and its hyperglycosylated analogue Darbepoetin alfa are known to be misused by athletes. The drugs can be detected in urine by isoelectric focusing, because the pattern of their glycans differs from that of endogenous EPO. However, doping control is becoming much more difficult, as various novel erythropoiesis stimulating agents (ESAs) are - or are to come - on the market. Gene-activated EPO (Epoetin delta) from human fibrosarcoma cells (HT-1080) has been approved in the European Union. rHuEPO (Epoetin omega) produced in transformed baby hamster kidney (BHK) cells is also available. ESAs to come include Biosimilars of the established Epoetins, long-acting pegylated Epoetin beta (CERA), EPO fusion proteins, synthetic erythropoiesis stimulating protein (SEP) and peptidic (Hematide) as well as non-peptidic EPO mimetics. Furthermore, small orally active drugs that are capable of stimulating endogenous EPO production are in preclinical or clinical trials. These include stabilizers of the hypoxia-inducible transcription factors (HIF) that bind to the EPO gene enhancer and GATA inhibitors which prevent GATA from suppressing the EPO gene promoter. It is crucial to inform the athletes and their supporting staff on the potential health risks.

Key words: *erythropoietin, erythropoiesis, recombinant DNA-technology, exercise, doping*

Introduction

In endurance sports – such as long-distance running, cycling and skiing – performance relies on an adequate O_2-supply to the heart and skeletal muscles. Hence, the rate of maximal O_2-uptake ($\dot{V}O_2$ max) is an important determinant of the aerobic power. $\dot{V}O_2$ max correlates with the O_2-carrying capacity of the blood. The O_2-capacity of the blood increases with the blood hemoglobin concentration (Hb). Thus, within certain limits, higher Hb levels are associated with improved performance. Apart from the O_2-content of the arterial blood the aerobic power increases with the maximal heart rate and stroke volume, and the myoglobin content and activities of mitochondrial enzymes of the muscles (Fig. 1).

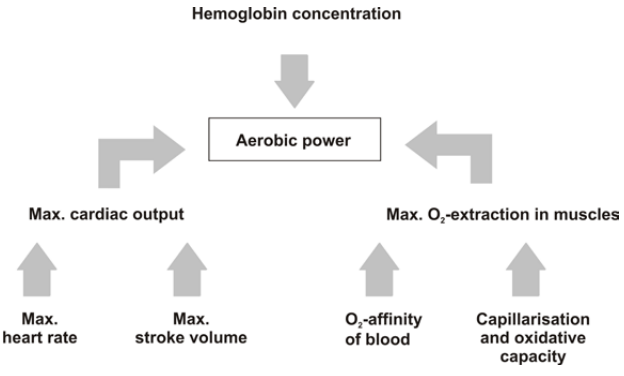

Fig. 1. *Parameters determining the aerobic power of a person (modified from (68))*

Red blood cell production is regulated by the glycoprotein hormone erythropoietin (EPO). EPO gene expression is controlled by feedback mechanisms involving the tissue O_2-pressure (pO_2), which depends on the Hb concentration, the arterial pO_2, the O_2-affinity of the blood and the rate of blood flow. After birth the kidney becomes the main EPO producing organ. EPO gene expression is under the control of several transcription factors (Fig. 2). The 5' promoter possesses GATA binding sites (1). GATA-2 has been reported to inhibit EPO gene expression (2-4). In addition, the EPO promoter is thought to be suppressed by NF-κB (5). The hypoxia-inducible EPO enhancer which is a 50-base pair element located 3' of the EPO gene contains at least two transcription factor binding sites. The proximal site of the EPO enhancer binds the hypoxia-inducible factors-1 or -2 (HIF-1 or -2) which

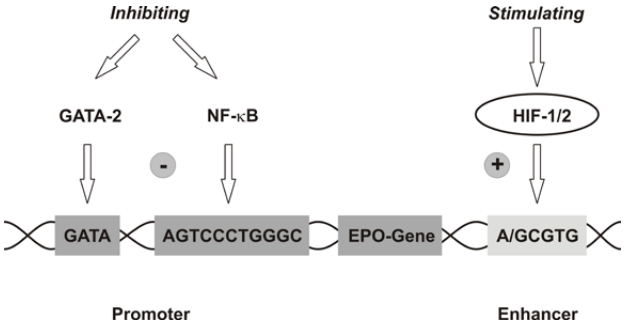

Fig. 2. *Transcriptional factors that suppress (-) the EPO promoter or activate (+) the EPO enhancer, respectively*

act as transcriptional activators (6). The distal site of the enhancer consists of a direct repeat of two nuclear hormone receptor half sites for hepatocyte nuclear factor-4α (HNF-4α) which cooperates with HIF (7).

Although GATA-2 binding to the EPO promoter is reduced on hypoxic stress (2, 3), the main mechanism by which hypoxia stimulates the expression of the EPO gene is binding of HIF. Recent studies indicate that HIF-2 (rather than HIF-1) is the primary transcription factor inducing EPO expression in hypoxia (8, 9). The HIFs are heterodimeric proteins composed of an O_2-labile α-subunit and a constitutive β-subunit (10). The C-terminus of the HIF-α subunits possesses O_2-dependent degradation domains (O-DDD), which contain O_2-sensitive prolyl and asparaginyl residues. Catalyzed by specific prolyl-4-hydroxylases (HIF-PHD) distinct prolyl residues (Pro^{402} and Pro^{564} in human HIF-1α, Pro^{405} and Pro^{531} in human HIF-2α) are hydroxylated in the presence of O_2, Fe^{2+} and 2-oxoglutarate (for references see Bruegge et al. (11)). Prolyl hydroxylated HIF-α combines with the von Hippel-Lindau tumor suppressor protein (pVHL) to form complexes that are polyubiquitinated by an E3-ligase and undergo immediate proteasomal degradation. The transcriptional activity of the HIFs is suppressed by another O_2-dependent hydroxylation, namely at Asn^{803} in human HIF-1α and Asn^{847} in human HIF-2α. This reaction is catalyzed by a HIF-α specific asparaginyl hydroxylase that is also termed „factor-inhibiting HIF-1" (FIH-1) (12, 13). On asparaginyl hydroxylation the binding of the co-activator CBP/p300 to the transactivating domain of HIF-α is prevented (12, 14, 15). Like the PHDs, FIH-1 is an Fe^{2+} containing and α-oxoglutarate requiring dioxygenase.

The HIF transcription factors do not only activate the EPO gene. More than 100 genes have been identified that are induced by binding of HIF to hypoxia-response elements (HRE), including the genes encoding the glucose transporters 1 and 3, several glycolytic enzymes and vascular endothelial growth factor (VEGF) (16, 17). Little is known about the consequences of the activation of these genes by HIFs in regard to physical performance.

Because the tissue pO_2 is the controlled variable, EPO gene expression will not only be stimulated when the O_2 capacity of the blood is lowered (anemia) but also on residence at high altitude (hypoxemia). On ascent to altitude serum EPO reaches peak values after about one day and then falls to a new plateau that is moderately above sea-level value (18, 19). The stimulation of EPO production by hypoxemia has been used by sportsmen to improve performance. Living and/or training at altitude may result in augmented erythropoiesis, a lowered O_2-affinity of red blood cells due to an increase in erythrocytic 2,3-disphosphoglycerate levels, and an improved oxidative capacity of muscles (20-23). Ethically questionable, high altitude residence can be mimicked at sea-level by living in tents or rooms with reduced O_2-concentrations (for references, see (24)). However, not all authors have confirmed that living under conditions of moderate hypoxia leads to increases in Hb and $\dot{V}O_2max$ (25).

Physiology of EPO

Tissue hypoxia is the main stimulus of EPO production in the kidneys and other organs (for references see (26)). In situ hybridization studies have identified a subgroup of peritubular fibroblasts as the site of EPO gene expression in the kidney (27). Human EPO is an acidic glycoprotein with a molecular mass of 30.4 kDa. Its peptide chain consists of 165 amino acids which form two bisulfide bridges. The carbohydrate portion (40% of the molecule) consists of 3 tetra-antennary N-linked (at Asn^{24}, Asn^{38} and Asn^{83}) and one small O-linked (at Ser^{126}) glycans (28). Like other plasma glycoproteins EPO circulates as a pool of isoforms that differ in glycosylation and biological activity (29-31). The N-glycans, which possess terminal sialic acid residues, are essential for the *in vivo* biological activity of EPO (32). De-sialylated EPO is rapidly removed from the circulation. *Vice versa,* the introduction of additional N-glycans into recombinant EPO by genetic engineering results in EPO analogues with prolonged *in vivo* survival (33).

The principal targets of EPO are erythrocytic progenitors in the bone marrow, particularly the colony-forming units-erythroid (CFU-Es). The erythrocytic human EPO receptor (EPO-R) is a 484 amino acid membrane protein. Its calculated mass of 52.6 kDa is increased to about 60 kDa due to glycosylation and phosphorylation (34). One EPO molecule binds to two EPO-R molecules which constitute a homodimer (35, 36). With a view to the novel erythropoiesis stimulating agents (ESA) it is noteworthy that the affinity of EPO analogues for EPO-R decreases with the degree of glycosylation of EPO (37). On EPO binding to its receptor intracellular signaling pathways are activated that prevent the programmed cell death of the erythrocytic progenitors and promote their proliferation and differentiation.

The normally low concentration of EPO enables only a small percentage of erythrocytic progenitors to survive and to proliferate (38). However, when the concentration of EPO rises in blood, either endogenously or following the administration of ESA, more CFU-Es escape from apoptosis and give rise to morphologically identifiable proerythroblasts and normoblasts. The developmental time from a CFU-E to its reticulocytic progeny is several days and involves 4-6 cell devisions. Thus, it takes about 3-4 days before significant reticulocytosis becomes apparent after an acute increase in plasma EPO. The action of EPO on

the myeloid erythrocytic progenitors is augmented by other hormones, f.e. by androgens (39). Androgenic steroids were earlier used clinically for treatment of severe anemia (40).

EPO therapy in renal failure

Lack of EPO is the primary reason for the development of anemia in chronic kidney disease (CKD). Recombinant human EPO (rHuEPO) was introduced as an antianemic drug for treatment of CKD patients 20 years ago (41, 42). The first-generation rHuEPO preparations (Epoetin alfa and Epoetin beta) have been engineered in cultures of transformed Chinese hamster ovary (CHO) cells that carry cDNA encoding human EPO (43). In addition, rHuEPO (Epoetin omega) engineered in baby hamster kidney (BHK) cell cultures has been used clinically in some Eastern European, Central American and Asian countries (44-46). In view of the relationship between the number and integrity of the N-glycans and the *in vivo* stability of EPO a CHO-cell derived hyperglycosylated rHuEPO analogue (Darbepoetin alfa) has been developed, which is increasingly administered to CKD patients. This compound possesses two extra N-linked carbohydrate chains as a result of site-directed mutagenesis for exchange of five amino acids. Compared to the Epoetins, which have a plasma half-life of 6-8 h, Darbepoetin alfa has a 3-fold longer half-life (37). The biological activity of 200 U (units) *per* µg rHuEPO peptide core corresponds to that of 1 µg Darbepoetin alfa peptide (47). In addition to the anemia of CKD, the anemias associated with cancer, myelodysplastic syndromes, bone marrow transplantation, autoimmune diseases, AIDS, hepatitis C and heart failure can be prevented by treatment with rHuEPO or Darbepoetin alfa (48). In addition, the drugs have been administered in the surgical setting to stimulate erythropoiesis in phlebotomy programs for autologous re-donation or for correction of a pre- or postoperative state of anemia. Note that it is recommended that the doses of ESAs should be adjusted in general for each patient (including renal failure patients, cancer patients receiving chemotherapy and patients receiving ESAs pre-operatively for reduction of allogeneic red blood cell transfusion) to achieve and maintain the lowest Hb level sufficient to avoid the need for red blood cell transfusion and not exceed 120 g/l. At higher Hb levels, ESA therapy may be associated with an increased risk for serious cardiovascular events (thromboembolism) and death.

Blood doping

According to the World Anti-Doping Agency (WADA) blood doping is the misuse of certain techniques and/or substances to increase one's red blood cell mass, which allows the body to transport more O_2 to muscles and therefore increase stamina and performance. Indeed, it has been known for decades that induced erythrocythemia will increase aerobic work capacity. F.e., almost 30 years ago Buick et al. (49) showed in a double-blind design that autologous re-infusion of approximately 900 ml of freeze-preserved blood increases $\dot{V}O_2max$ and running time to exhaustion in highly trained runners. Likewise, it has been shown that re-infusion of autologous blood stored in a refrigerator for 4 weeks after phlebotomy significantly increases performance in cross-country skiing (50). Clearly, the transfusion of blood to improve endurance performance of athletes during training or competition is forbidden (51). While homologous blood doping (transfusion of compatible blood taken from another person) is detectable by flow cytometry ((52); the test was implemented at the 2004 Summer Olympic Games in Athens), autologous blood doping (transfusion of one's own blood) cannot be detected at present by direct measures. However, the donor of red cells stored for autologous re-transfusion can be identified by comparative blood group-typing and other immunological markers. The apparent recent resurgence of blood transfusion for doping is a likely consequence of the introduction of the method for demonstration of rHuEPO in urinary samples in 2000 (53). Attempts may also be made to increase the O_2-capacity of the blood by use of hemoglobin-based O_2 carriers (HBOCs). However, HBOCs can be detected by electrophoretic methods (54) or size-exclusion HPLC (55).

Immediately after rHuEPO became available as an erythropoiesis-stimulating drug it has been imputed to be abused by athletes in aerobic sports (56, 57). There is suspicion that rHuEPO-induced erythrocytosis caused the deaths of 18 world-class Dutch and Belgian cyclists (57), although it has remained unproven that they were truly treated with rHuEPO. Blood viscosity and cardiac afterload increase with increasing hematocrit (Hct), while cerebral blood flow decreases (58). The main risks of erythrocytosis include heart failure, myocardial infarction, sizes, peripheral thromboembolic events and pulmonary embolism. The risks are raised during the competition when the blood viscosity increases further due to intensified perspiration and the shift of fluid from the intravascular into the interstitial space (for references, see (59)). The fact that the above mentioned cases of death of cyclists suspected of rHuEPO doping did not occur during exercise but during periods of physical inactivity does not militate against the detrimental effect of erythrocytosis, because blood flow in the microcirculation will slow down during physical inactivity thereby favoring the development of thrombi. The risk to promote tumor growth by EPO doping has been considered (60), although clinical evidence supporting this fear is missing.

A study in healthy male students in physical education who were moderately to well trained has shown that a low dose of rHuEPO (about 30 U/kg) injected subcutaneously three times a week increases $\dot{V}O_2$ max and physical performance, measured as time to exhaustion on a standard treadmill running test, along with the increase in Hb and Hct (61). These findings have been confirmed in other studies (62-65). rHuEPO treatment increases red cell mass in healthy humans, while blood plasma volume decreases, with both effects contributing to the elevation in Hb (66). For the most part, rHuEPO doping will be more effective than hypoxia exposure (67). In addition, a psychological investigation has shown that the perception of increased physical condition can lead to a stronger commitment to training. The authors have pointed out that the administration of rHuEPO is associated with a potentially dangerous hedonic effect linked to endurance training (65).

It should be noted that physical exercise *per se* does not have a major influence on circulating EPO levels as has been studied in various disciplines (for references see (68)). Despite the lack of an increase in EPO production in response to acute physical work, the number of reticulocytes may increase within 1 to 2 days after exercise (69). This reaction is likely caused by stress hormones such as catecholamines and cortisol which stimulate the release of young red blood cells from bone marrow. Note that Hb levels and Hct are often below normal in athletes (59). This so-called „sports anemia" is a pseudoanemia as it results from an increase in the blood plasma volume.

The exclusion from competition of athletes with abnormally high Hb or Hct values by some sport organizations (f.e.: UCI, FIS, ISU, IBU) is justified on medical prophylactic grounds. However, it is by no means proof of rHuEPO doping, because Hb levels in unmanipulated persons often exceed the limits set by sport organizations (see Fig. 3). In addition, Fig. 3 shows that there is a large variation in the normal levels of Hb and serum EPO in healthy humans.

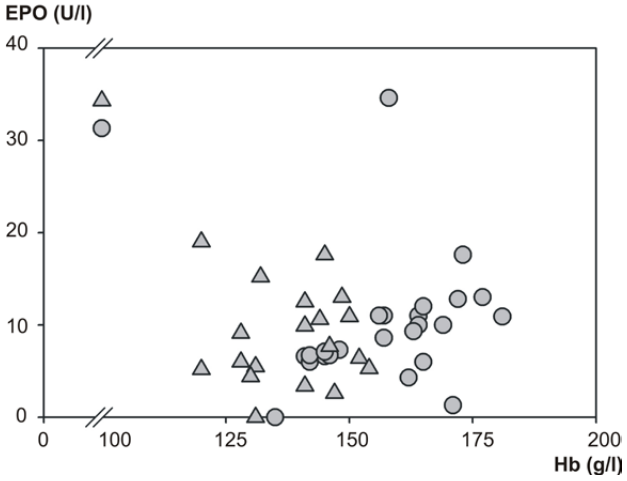

Fig. 3. *Serum EPO related to blood Hb concentrations in healthy humans (n = 43, laboratory staff (125))*

Detection of Epoetin and Darbepoetin doping

An indirect screening method for inspection of the misuse of Epoetin or Darbepoetin by athletes has been established which is based on the values of Hct, reticulocytes, macrocytes and the concentrations of circulating EPO and soluble tranferrin receptor (70-72). If the result provides grounds for suspicion, misuse can be proven by direct demonstration of the drugs in urine samples. The use of charge differences between recombinant and endogenous EPO in doping control was first proposed by Wide et al. (73). In the current procedure, about 20 ml urine is approximately 1000-fold concentrated and depleted of small molecules by ultrafiltration. The samples are subjected to isoelectric focusing (pH 2 - 6) followed by immunodetection (53). Non-specific interaction with other urinary proteins is minimized by an additional Western blotting step, whereby the primary monoclonal anti-EPO antibody (AE7A5) is transferred to a new membrane, which is then incubated with labeled anti-mouse immunoglobulin secondary antibody (74). Technical details of the procedure are described in a WADA document (75).

The EPO isoforms pattern of urine from untreated control subjects exhibits about 10 bands in the range pI 3.77 – 4.70 on immunoblots. Blots from subjects treated with Epoetin alfa, Epoetin beta or Epoetin omega contain more basic bands. Interestingly, when 14 frozen samples of urine with relatively high concentrations of immunoreactive EPO from participants in the Tour de France 1998 were subjected to isoelectric focusing after the method was newly developed, all of the samples exhibited a banding pattern typical of rHuEPO (53). Darbepoetin alfa migrates more in the acidic range than endogenous EPO (76, 77). The computer program GASepo (78), which is freely available for anti-doping laboratories, enables it to standardize the results by means of image segmentation. Methodological weaknesses of the detection of recombinant ESA have been described elsewhere (79-81). To overcome some of the problems (such as time-consuming urine sample preparation, low sample load capacity and non-specific binding) an improved concentration technique in combination with 2D electrophoresis has been developed (82). A novel problem is suspected adding of proteases by athletes to their urinary samples, which destroys the erythropoietic proteins. The detection of exogenous proteases is possible by mass spectrometry.

It has been suggested to establish individual „Hematologic passports" for elite athletes which would enable one to detect sudden unphysiological changes in hematologic parameters. However, whether such passports, which also include data on the total Hb mass as determined by CO re-breathing (83), is useful in practice still needs to be clarified. The significance

of such data in law is questionable. In addition, this procedure does not appear appropriate in leisure sports and in elite sport disciplines, in which the competitors have a short history until success.

Novel Epoetins

The WHO Expert Committee for International Nonproprietary Names (INN) defines „Epoetins" as products that are characterized by an amino acid sequence similar to that of endogenous EPO (84). Greek letters are added to differentiate between compounds varying in the glycosylation pattern.

Epoetin delta is a novel recombinant EPO for treatment of CKD patients (85, 86) that was approved by the European Medicines Agency (EMEA) in 2002 and first marketed, in Germany, in 2007. Epoetin delta is engineered in human fibrosarcoma cell cultures (line HT-1080). The product is also called gene activated EPO (GA-EPO) because the expression of the native human EPO gene is activated by transformation of the cells with the cytomegalovirus (CMV) promoter (87). In contrast to CHO or BHK cell-derived rHuEPO, Epoetin delta does not possess N-glycolylneuraminic acid (Neu5Gc) because – in contrast to other mammals including great apes – humans are genetically unable to produce Neu5Gc due to an evolutionary mutation (88). Nevertheless, it is unlikely that the behavior of Epoetin delta on isoelectric focusing is identical to that of native human urinary EPO because the structure of N-glycans is not only species- but also tissue-specific. In addition, the pattern of isoforms is influenced by the environmental respectively cell culture conditions and the purification procedures applied. In fact, earlier studies have shown that rHuEPO from a human lymphoblastoid cell line (RPMI 1788) transfected with the human EPO gene possesses N-glycans with unusual characteristics (89). Support for the expectation that Epoetin delta doping may be detectable comes also from observations following homologous EPO gene transfer. *In vivo* EPO gene transfer in skeletal muscle in macaques results in EPO which differs in isoelectric pattern from the endogenous EPO of the animals as demonstrated by isoelectric focusing (90).

Since the patents of Epoetin alfa and Epoetin beta have expired recently in several countries and because the market for rHuEPO is very lucrative, copies of the established rHuEPO preparations are expected to be marketed soon, respectively are already on the market. These products are named „Biosimilars" in the EU and „Follow-on Biologics" in the USA. Outside the EU and the USA copy rHuEPOs are already produced by companies other than the innovators and clinically used as anti-anemic drugs. F.e., the therapeutic efficacy of a CHO cell-derived rHuEPO produced in Havana, Cuba, has been proven (91). Apart from this single report, however, little information is available on the structure, pharmacodynamics and pharmacokinetics of copy rHuEPO preparations. Because the glycosylation pattern of rHuEPO depends on the cell line, the culture conditions and the product purification procedures it is likely that all copy rHuEPOs differ with respect to the structure of their N-glycans. Indeed, when 11 samples of Epoetin products marketed outside of Europe and the USA were obtained from 8 different manufacturers were investigated for their behavior on isoelectric focusing major differences between the products of different manufacturers as well as between batches of products of the same manufacturer were detected (92). Clearly, the availability of rHuEPO preparations with isoforms that are more acidic as well as such with isoforms that are more basic than endogenous EPO will pose major problems with respect to doping analysis, in particular if the products are used at low dosing and in combination. In addition, it is reasonable to imagine irresponsible athletes and trainers who have made use of counterfeit Epoetin preparations, which have cropped up in the USA (93) and elsewhere. Subjects prone to misuse copy or counterfeit Epoetins should be aware that such products do not always meet the self-declared specifications and the common purity standards (94).

Another new Epoetin derivate in clinical trials is CERA (Continuous Erythropoiesis Receptor Activator, Ro 50-3821). The molecular mass (60 kDa) of CERA is about twice that of the Epoetins, because a methoxy-polyethylene glycol polymer (PEG) is integrated *via* amide bonds between the N-terminal amino group of alanine (Ala^1) and the ε-amino groups of lysine (Lys^{45} or Lys^{52}) by means of a succinimidyl butanoic acid linker (95). As it is known from pharmacokinetic studies of other pegylated therapeutic proteins (96, 97), the half-life of circulating CERA is prolonged (to about 6 days) compared to that of the conventional Epoetins alfa and beta (98). Details of the sites and mechanisms of the clearance of CERA still need to be described. Apart from CERA, pegylated Epoetin alfa (99) and a pegylated rHuEPO analogue (100) have been tested for their efficacy in experimental animals.

Several other EPO-like molecules and derivatives are in preclinical or clinical trials (Table 1). (i) A hyperglycosylated analogue of Darbepoetin alfa (AMG 114) with a prolonged half-life is tested clinically. (ii) An interesting novel product is SEP (Synthetic Erythropoiesis Protein, 50 kDa) which is chemically synthesized as 166 amino acid protein (including the Arg^{166} that is cleaved before human EPO is secreted from cells) with covalently bound polymer moieties (101). The half-life of circulating SEP is probably longer than that of the Epoetins. The erythropoietic effect of SEPs has been shown to vary in experimental animals depending on the number and type of the at-

tached polymers (102). (iii) Recombinant EPO fusion proteins have been expressed which contain additional peptides at the carboxy-terminus to increase *in vivo* survival (103). (iiii) Large EPO fusion proteins (76 kDa) have been designed which are derived from cDNA encoding two human EPO molecules linked by small flexible polypeptides (104, 105). (iiiii) Still another approach is the genetic fusion of EPO with the Fc region of human IgG (106). Interestingly, an Fc-EPO fusion protein has been successfully administered in a phase I trial to human volunteers as an aerosol (107). With a view to the possible routes of administration, it should be noted that rHuEPO has been applicated by ultrasound-mediated transdermal uptake to humans (108) and by oral route in liposomes to rats (109).

Table 1. *Novel drugs for stimulating erythropoiesis*

Protein-based ESAs
Epoetin alfa, Epoetin beta, Epoetin omega
Darbepoetin alfa
Epoetin delta
Copy rHuEPOs, biosimilars and counterfeit products
CERA
SEP
EPO fusion proteins
Small molecule EPO mimetics
Peptides
Non-peptides
HIF stabilisers
Cobalt
α-Ketoglutarate competitors

EPO mimetics

About 10 years ago several small bisulfide-linked cyclic peptides composed of about 20 amino acids were identified by random phage display technology which are unrelated in sequence to EPO but bind to the EPO receptor and are erythropoietically active (35, 36). Subsequent studies showed that the potency of the EPO mimetic peptides (EMP) could be greatly increased by covalent peptide dimerization with a PEG linker (110). A potent EMP has been chosen to develop Hematide, a pegylated synthetic dimeric peptidic ESA, which stimulates erythropoiesis in experimental animals (111). The half-life of Hematide in monkeys ranges from 14 h to 60 h depending on the applicated dose (111). A phase I study in healthy male volunteers has shown that single injections of Hematide cause dose-dependent increases in the concentration of reticulocytes and Hb (112). Hematide is currently in phase II of its clinical trial program. Although Hematide can be detected by appropriate ELISA no data are available demonstrating Hematide in human urine.

Several non-peptide molecules capable of mimicking the effects of EPO have also been identified (113, 114) but the potential of these compounds to stimulate erythropoiesis in humans still needs to be proven.

HIF activators

Under normoxic conditions EPO gene expression is suppressed physiologically because the hypoxia-inducible transcription factors (HIF) are inactivated due to HIF-α prolyl and asparaginyl hydroxylation. The HIF-α hydroxylases do not only require O_2 for their catalytic action but also Fe^{2+} and 2-oxoglutarate (for references, see (11)). Accordingly, HIF-α hydroxylation can be prevented by iron depletion or by the application of 2-oxoglutarate analogues (Fig. 4).

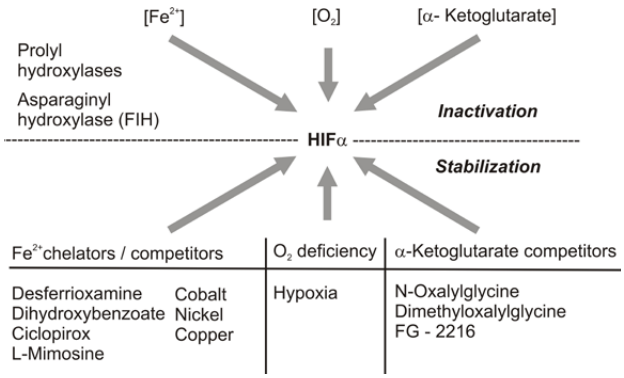

Fig. 4. *Compounds inducing endogenous EPO expression by inhibiting HIF-α hydroxylation in normoxia*

stabilize HIF-α (115-118) and promote EPO expression in cell cultures (117-119) as well as in humans *in vivo* (119, 120). However, iron chelators are not suited for stimulation of red cell production in humans in the long term because iron is required for heme synthesis. HIF-dependent EPO gene expression in normoxia can also be induced by divalent transition metals such as cobalt or nickel which – at leased partially – exert their action by displacing Fe^{2+} from the HIF-α hydroxylases (121, 122). In addition, cobalt appears to bind to HIF-2α directly, thereby preventing the normoxic degradation of HIF-2α (123). The stimulation of EPO production and erythropoiesis by cobaltous ions has been known for many years (124). Actually, the international EPO standard was originally calibrated against cobalt, with 1 unit (U) of EPO producing the same erythropoiesis-stimulating response in experimental animals as 5 μmol cobaltous chloride (for references see (125)). Prior to the availability of rHuEPO cobalt was used to treat anemic patients (126). Note that the use of cobalt as an anti-anemic drug is today obsolete because of its toxicity. Nevertheless, it has been speculated that

some athletes may misuse cobalt to stimulate EPO production (127).

With a view to doping, the 2-oxoglutarate analogues which stabilize HIF-α in normoxia („HIF-stabilizers") are a major problem. Many of these compounds were earlier tested *in vitro* (128) and *in vivo* (129, 130) for their inhibitory action on collagen prolyl 4-hydroxylases which also need 2-oxoglutarate as cofactor. The primary purpose of the initial studies was to develop drugs for treatment of fibrotic diseases (131, 132). Among the HIF-stabilizers are simple molecules like N-oxalylglycine, ethyl-3,4-dihydroxybenzoate or L-mimosine which are easy to synthetize and can be taken orally. 2-Oxoglutarate analogues clearly stimulate erythropoiesis *in vivo* (133). HIF-stabilizers have already been administered to healthy control subjects (134) and to patients with CKD (135) in clinical trials investigating novel strategies for the treatment of anemia. It should be noted, however, that the application of 2-oxoglutarate analogues to induce EPO expression will result in the expression of a great number of other genes which may result in serious unwanted effects. A major concern is the promotion of tumor growth.

Conclusion

In their worth reading reappraisal of the abuse of drugs by athletes Duntas and Parisis (136) have cited the ancient Greek saying „None so wretched as the competitor who wins victory through cheating". Yet the authors have also conceded that the commercialization has progressively changed the spirit of sport, as the desire to win at any cost has overcome all ethical and medical considerations. Success in elite sports gives promise of fame and financial rewards. Moreover, trainers and sport officials press their protégées for victories, and we (the spectators) long for heroes. There is little doubt that the novel erythropoiesis stimulating drugs will be picked up in attempts to improve performance. Unfortunately, misuse of drugs in sports is not restricted to elite sports but has also gained entry to leisure sports. Here, an additional health problem relates to the fact that amateur athletes will often act without medical control.

With several novel erythropoietic drugs being on the market doping control has become very difficult, not to say hopeless. The new drugs include various kinds of Epoetins, biosimilars, mutated EPO analogues, long-acting pegylated Epoetins and EPO analogues, EPO fusion proteins and peptidic as well as non-peptidic EPO mimetics. Compounds are at hand which act as stabilizers of hypoxia-inducible transcription factors (HIF) that bind to the EPO enhancer. GATA inhibitors are under development, which can be orally taken and prevent GATA from suppressing the EPO promoter (137;138). Because EPO signaling involves protein tyrosine phosphorylation, inhibitors of hemopoietic cell phosphatase (HCP) may also become available for misuse (139). The novel drugs are being developed primarily for therapeutic benefits, i.e. the alleviation of anemia in patients suffering from chronic renal failure or inflammatory or malignant diseases (140). Because there are not only major technical problems in detecting the drugs for proof of doping but also difficulties with respect to intention, moral and law, it is crucial to inform the athletes and their supporting staff of potential health risks, although this may be an act of „tilting at windmills".

Acknowledgement

Thanks are due to Ms. Lisa Zieske for expert secretarial help in the preparation of the manuscript.

References

1. Blanchard KL, Acquaviva AM, Galson DL, Bunn HF. Hypoxic induction of the human erythropoietin gene: cooperation between the promoter and enhancer, each of which contains steroid receptor response elements. Mol Cell Biol 1992; 12: 5373-85.
2. Imagawa S, Izumi T, Miura Y. Positive and negative regulation of the erythropoietin gene. J Biol Chem 1994; 269: 9038-44.
3. Imagawa S, Yamamoto M, Miura Y. Negative regulation of the erythropoietin gene expression by the GATA transcription factors. Blood 1997; 89: 1430-9.
4. Tabata M, Tarumota T, Ohmine K, Furukawa Y, Hatake K, Ozawa K, et al. Stimulation of GATA-2 as a mechanism of hydrogen peroxide suppression in hypoxia-induced erythropoietin gene expression. J Cell Physiol 2001; 186: 260-7.
5. La Ferla K, Reimann C, Jelkmann W, Hellwig-Bürgel T. Inhibition of erythropoietin gene expression signaling involves the transcription factors GATA-2 and NF-κB. FASEB J 2002;16: 1811-3.
6. Semenza GL, Nejfelt MK, Chi SM, Antonarakis SE. Hypoxia-inducible nuclear factors bind to an enhancer element located 3' to the human erythropoietin gene. Proc Natl Acad Sci USA 1991; 88: 5680-4.
7. Galson DL, Tsuchiya T, Tendler DS, Huang LE, Ren Y, Ogura T, et al. The orphan receptor hepatic nuclear factor 4 functions as a transcriptional activator for tissue-specific and hypoxia-specific erythropoietin gene expression and is antagonized by EAR3/COUP-TF1. Mol Cell Biol 1995; 15: 2135-44.
8. Warnecke C, Zaborowska Z, Kurreck J, Erdmann VA, Frei U, Wiesener M, et al. Differentiating the functional role of hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha (EPAS-1) by the use of RNA interference: erythropoietin is a HIF-2alpha target gene in Hep3B and Kelly cells. FASEB J 2004; 18: 1462-4.
9. Rankin EB, Biju MP, Liu Q, Unger TL, Rha J, Johnson RS, et al. Hypoxia-inducible factor-2 (HIF-2) regulates hepatic erythropoietin in vivo. J Clin Invest 2007; 117: 1068-77.
10. Wang GL, Semenza GL. Purification and characterization of hypoxia-inducible factor 1. J Biol Chem 1995;270: 1230-7.
11. Bruegge K, Jelkmann W, Metzen E. Hydroxylation of hypoxia-inducible transcription factors and chemical compounds targeting the HIF- hydroxylases. Curr Med Chem 2007; 14: 103-12.
12. Mahon PC, Hirota K, Semenza GL. FIH-1: a novel protein that interacts with HIF-1α and VHL to mediate repression of HIF-1 transcriptional activity. Genes Dev 2001; 15: 2675-86.
13. McNeill LA, Hewitson KS, Claridge TD, Seibel JF, Horsfall LE, Schofield CJ. Hypoxia-inducible factor asparaginyl hydroxylase (FIH-1) catalyses hydroxylation at the beta-carbon of asparagine-803. Biochem J 2002; 367: 571-5.

14. Lando D, Peet DJ, Whelan DA, Gorman JJ, Whitelaw ML. Asparagine hydroxylation of the HIF transactivation domain: a hypoxic switch. Science 2002; 295: 858-61.
15. Lando D, Peet DJ, Gorman JJ, Whelan DA, Whitelaw ML, Bruick RK. FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. Genes Dev 2002; 16: 1466-71.
16. White JR, Harris RA, Lee SR, Craigon MH, Binley K, Price T, et al. Genetic amplification of the transcriptional response to hypoxia as a novel means of identifying regulators of angiogenesis. Genomics 2004; 83: 1-8.
17. Manalo DJ, Rowan A, Lavoie T, Natarajan L, Kelly BD, Ye SQ, et al. Transcriptional regulation of vascular endothelial cell responses to hypoxia by HIF-1. Blood 2005; 105: 659-69.
18. Abbrecht PH, Littell JK. Plasma erythropoietin in men and mice during acclimatization to different altitudes. J Appl Physiol 1972; 32: 54-8.
19. Milledge JS, Cotes PM. Serum erythropoietin in humans at high altitude and its relation to plasma renin. J Appl Physiol 1985; 59: 360-4.
20. Berglund B. High-altitude training. Aspects of haematological adaptation. Sports Med 1992; 14: 289-303.
21. Mairbaurl H. Red blood cell function in hypoxia at altitude and exercise. Int J Sports Med 1994; 15: 51-63.
22. Selby GB, Eichner ER. Hematocrit and performance: the effect of endurance training on blood volume. Semin Hematol 1994; 31: 122-7.
23. Fulco CS, Rock PB, Cymerman A. Improving athletic performance: is altitude residence or altitude training helpful? Aviat Space Environ Med 2000; 71: 162-71.
24. Lippi G, Franchini M, Salvagno GL, Guidi GC. Biochemistry, physiology, and complications of blood doping: facts and speculation. Crit Rev Clin Lab Sci 2006; 43: 349-91.
25. Hahn AG, Gore CJ, Martin DT, Ashenden MJ, Roberts AD, Logan PA. An evaluation of the concept of living at moderate altitude and training at sea level. Comp Biochem Physiol A Mol Integr Physiol 2001; 128: 777-89.
26. Jelkmann W. Erythropoietin: structure, control of production, and function. Physiol Rev 1992;72: 449-89.
27. Bachmann S, Le Hir M, Eckardt KU. Co-localization of erythropoietin mRNA and ecto-5'-nucleotidase immunoreactivity in peritubular cells of rat renal cortex indicates that fibroblasts produce erythropoietin. J Histochem Cytochem 1993; 41: 335-41.
28. Sasaki H, Ochi N, Dell A, Fukuda M. Site-specific glycosylation of human recombinant erythropoietin: analysis of glycopeptides or peptides at each glycosylation site by fast atom bombardment mass spectrometry. Biochemistry 1988; 27: 8618-26.
29. Sherwood JB, Carmichael LD, Goldwasser E. The heterogeneity of circulating human serum erythropoietin. Endocrinology 1988; 122: 1472-7.
30. Sohmiya M, Kato Y. Molecular and electrical heterogeneity of circulating human erythropoietin measured by sensitive enzyme immunoassay. Eur J Clin Invest 2000; 30: 344-9.
31. Skibeli V, Nissen-Lie G, Torjesen P. Sugar profiling proves that human serum erythropoietin differs from recombinant human erythropoietin. Blood 2001; 98: 3626-34.
32. Weikert S, Papac D, Briggs J, Cowfer D, Tom S, Gawlitzek M, et al. Engineering Chinese hamster ovary cells to maximize sialic acid content of recombinant glycoproteins. Nat Biotechnol 1999; 17: 1116-21.
33. Elliott S, Lorenzini T, Asher S, Aoki K, Brankow D, Buck L, et al. Enhancement of therapeutic protein in vivo activities through glycoengineering. Nat Biotechnol 2003; 21: 414-21.
34. Elliott S, Busse L, Spahr C, Sinclair AM. Anti-EpoR antibodies detect a 59-kDa EpoR protein. Blood 2006; 108: 1109.
35. Livnah O, Stura EA, Johnson DL, Middleton SA, Mulcahy LS, Wrighton NC, et al. Functional mimicry of a protein hormone by a peptide agonist: the EPO receptor complex at 2.8 A. Science 1996; 273: 464-71.
36. Wrighton NC, Farrell FX, Chang R, Kashyap AK, Barbone FP, Mulcahy LS, et al. Small peptides as potent mimetics of the protein hormone erythropoietin. Science 1996; 273: 458-64.
37. Egrie JC, Browne JK. Development and characterization of novel erythropoiesis stimulating protein (NESP). Br J Cancer 2001; 84: 3-10.
38. Koury MJ, Bondurant MC. The molecular mechanism of erythropoietin action. Eur J Biochem 1992; 210: 649-63.
39. Mizoguchi H, Levere RD. Enhancement of heme and globin synthesis in cultured human marrow by certain 5 -H steroid metabolites. J Exp Med 1971; 134: 1501-12.
40. Besa EC. Hematologic effects of androgens revisited: an alternative therapy in various hematologic conditions. Semin Hematol 1994; 31: 134-45.
41. Winearls CG, Oliver DO, Pippard MJ, Reid C, Downing MR, Cotes PM. Effect of human erythropoietin derived from recombinant DNA on the anaemia of patients maintained by chronic haemodialysis. Lancet 1986; 2: 1175-8.
42. Eschbach JW, Egrie JC, Downing MR, Browne JK, Adamson JW. Correction of the anemia of end-stage renal disease with recombinant human erythropoietin. Results of a combined phase I and II clinical trial. N Engl J Med 1987; 316: 73-8.
43. Lin FK, Suggs S, Lin CH, Browne JK, Smalling R, Egrie JC, et al. Cloning and expression of the human erythropoietin gene. Proc Natl Acad Sci U S A 1985; 82: 7580-4.
44. Acharya VN, Sinha DK, Almeida AF, Pathare AV. Effect of low dose recombinant human omega erythropoietin (rHuEPO) on anaemia in patients on hemodialysis. J Assoc Physicians India 1995; 43: 539-42.
45. Bren A, Kandus A, Varl J, Buturovic J, Ponikvar R, Kveder R, et al. A comparison between epoetin omega and epoetin alfa in the correction of anemia in hemodialysis patients: a prospective, controlled crossover study. Artif Organs 2002; 26: 91-7.
46. Sikole A, Spasovski G, Zafirov D, Polenakovic M. Epoetin omega for treatment of anemia in maintenance hemodialysis patients. Clin Nephrol 2002; 57: 237-45.
47. Jelkmann W. The enigma of the metabolic fate of circulating erythropoietin (Epo) in view of the pharmacokinetics of the recombinant drugs rhEpo and NESP. Eur J Haematol 2002; 69: 265-74.
48. Henry DH, Bowers P, Romano MT, Provenzano R. Epoetin alfa. Clinical evolution of a pleiotropic cytokine. Arch Intern Med 2004; 164: 262-76.
49. Buick FJ, Gledhill N, Froese AB, Spriet L, Meyers EC. Effect of induced erythrocythemia on aerobic work capacity. J Appl Physiol 1980; 48: 636-42.
50. Berglund B, Hemmingson P. Effect of reinfusion of autologous blood on exercise performance in cross-country skiers. Int J Sports Med 1987; 8: 231-3.
51. Simon TL. Induced erythrocythemia and athletic performance. Semin Hematol 1994; 31: 128-33.
52. Nelson M, Popp H, Sharpe K, Ashenden M. Proof of homologous blood transfusion through quantification of blood group antigens. Haematologica 2003; 88: 1284-95.
53. Lasne F, de Ceaurriz J. Recombinant erythropoietin in urine. Nature 2000; 405: 635.
54. 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. Clinical Chemistry 2004; 50: 410-5.
55. Varlet-Marie E, Ashenden M, Lasne F, Sicart MT, Marion B, de CJ, et al. Detection of hemoglobin-based oxygen carriers in human serum for doping analysis: confirmation by size-exclusion HPLC. Clin Chem 2004; 50: 723-31.
56. Spalding BJ. Black-market biotechnology: athletes abuse EPO and HGH. Biotechnology N Y 1991; 9: 1050-3.
57. Eichner E. Better dead than second. J Lab Clin Med 1992; 120: 359-60.
58. Thomas DJ, Marshall J, Russell RW, Wetherley-Mein G, du-Boulay GH, Pearson TC., et al. Effect of haematocrit on cerebral blood-flow in man. Lancet 1977; 2: 941-3.
59. Szygula Z. Erythrocytic system under the influence of physical exercise and training. Sports Med 1990; 10: 181-97.

60. Tentori L, Graziani G. Doping with growth hormone/IGF-1, anabolic steroids or erythropoietin: is there a cancer risk? Pharmacol Res 2007; 55(5): 359-69.
61. Ekblom B, Berglund B. Effect of erythropoietin administration on maximal aerobic power in man. Scand J Med Sci Sports 1991; 1: 88-93.
62. Audran M, Gareau R, Matecki S, Durand F, Chenard C, Sicart M, et al. Effects of erythropoietin adminstration in training athletes and possible indirect detection in doping control. Med Sci Sports Exerc 1999; 5: 639-45.
63. Birkeland KI, Stray-Gundersen J, Hemmersbach P, Hallen J, Haug E, Bahr R. Effect of rhEPO administration on serum levels of sTfR and cycling performance. Med Sci Sports Exerc 2000; 32: 1238-43.
64. 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: 231-8.
65. Ninot G, Connes P, Caillaud C. Effects of recombinant human erythropoietin injections on physical self in endurance athletes. J Sports Sci 2006; 24: 383-91.
66. Lundby C, Thomsen JJ, Boushel R, Koskolou M, Warberg J, Calbet JA, et al. Erythropoietin treatment elevates haemoglobin concentration by increasing red cell volume and depressing plasma volume. J Physiol 2007; 578: 309-14.
67. 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: 831-7.
68. Jelkmann W. Erythropoietin. J Endocrinol Invest 2003; 26: 832-7.
69. Schmidt W, Maassen N, Trost F, Boning D. Training induced effects on blood volume, erythrocyte turnover and haemoglobin oxygen binding properties. Eur J Appl Physiol Occup Physiol 1988; 57: 490-8.
70. Parisotto R, Ashenden MJ, Emslie KR, Gore CJ, Howe C, Kazlauskas R, et al. Detection of recombinant human erythropoietin abuse in athletes utilizing markers of altered erythropoiesis. Haematologica 2001; 86: 128-37.
71. Casadevall N. Antibodies against rHuEPO: native and recombinant. Nephrol Dial Transplant 2002; 17 Suppl 5: 42-7.
72. Gore CJ, Parisotto R, Ashenden MJ, Stray-Gundersen J, Sharpe K, Hopkins W, et al. Second-generation blood tests to detect erythropoietin abuse by athletes. Haematologica 2003; 88: 333-44.
73. 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: 1569-76.
74. Lasne F. Double-blotting: a solution to the problem of non-specific binding of secondary antibodies in immunoblotting procedures. J Immunol Methods 2001; 253: 125-31.
75. WADA. Harmonization of the method for the identification of Epoetin alfa and beta (EPO) and Darbepoetin alfa (NESP) by IEF-double blotting and chemiluminescent detection. WADA Technical Document No.: TD2004EPO. 2004. www.wada-ama.org/rtecontent/document/td2004epo_en.pdf.
76. 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: 119-26.
77. Catlin DH, Breidbach A, Elliott S, Glaspy J. Comparison of the isoelectric focusing patterns of darbepoetin alfa, recombinant human erythropoietin, and endogenous erythropoietin from human urine. Clin Chem 2002; 48: 2057-9.
78. Bajla I, Hollander I, Minichmayr M, Gmeiner G, Reichel C. GASepo-a software solution for quantitative analysis of digital images in Epo doping control. Comput Methods Programs Biomed 2005; 80: 246-70.
79. Franke WW, Heid H. Pitfalls, errors and risks of false-positive results in urinary EPO drug tests. Clin Chim Acta 2006; 373: 189-90.
80. Beullens M, Delanghe JR, Bollen M. False-positive detection of recombinant human erythropoietin in urine following strenuous physical exercise. Blood 2006; 107: 4711-3.
81. Lasne F. No doubt about the validity of the urine test for detection of recombinant human erythropoietin. Blood 2006; 108: 1778-9.
82. Khan A, Grinyer J, Truong ST, Breen EJ, Packer NH. New urinary EPO drug testing method using two-dimensional gel electrophoresis. Clin Chim Acta 2005; 358: 119-30.
83. Schmidt W, Prommer N. The optimised CO-rebreathing method: a new tool to determine total haemoglobin mass routinely. Eur J Appl Physiol 2005; 95: 486-95.
84. World Health Organization. International nonproprietary names (INN) for biological and biotechnological substances. World Health Organization; 2006. Report No.: INN Working Document 05.179.
85. Spinowitz BS, Pratt RD. Epoetin delta is effective for the management of anaemia associated with chronic kidney disease. Curr Med Res Opin 2006; 22: 2507-13.
86. Kwan JT, Pratt RD. Epoetin delta, erythropoietin produced in a human cell line, in the management of anaemia in predialysis chronic kidney disease patients. Curr Med Res Opin 2007; 23: 307-11.
87. The Court Service - Court of Appeal - Civil Judgment. In TKT's technology, those cells were designated as R223 cells. Neutral Citation Number: [2002] EWCA Civ. 1096. 2002. www.hmcourts-service.gov.uk/judgmentsfiles/j1329/Kirin_v_Hoechst.htm.
88. Tangvoranuntakul P, Gagneux P, Diaz S, Bardor M, Varki N, Varki A, et al. Human uptake and incorporation of an immunogenic nonhuman dietary sialic acid. Proc Natl Acad Sci U S A 2003 Oct 14 100; 12045-50.
89. Cointe D, Beliard R, Jorieux S, Leroy Y, Glacet A, Verbert A, et al. Unusual N-glycosylation of a recombinant human erythropoietin expressed in a human lymphoblastoid cell line does not alter its biological properties. Glycobiology 2000; 10: 511-9.
90. Lasne F, Martin L, de Ceaurriz J, Larcher T, Moullier P, Chenuaud P. „Genetic Doping" with erythropoietin cDNA in primate muscle is detectable. Mol Ther 2004; 10: 409-10.
91. Perez-Oliva JF, Casanova-Gonzalez M, Garcia-Garcia I, Porrero-Martin PJ, Valenzuela-Silva CM, Hernandez-Montero T, et al. Comparison of two recombinant erythropoietin formulations in patients with anemia due to end-stage renal disease on hemodialysis: a parallel, randomized, double blind study. BMC Nephrol 2005; 6: 5.
92. Schellekens H. Biosimilar epoetins: how similar are they? Eur J Hosp Pharm 2004; 3: 43-7.
93. http://www.fda.gov/medwatch/SAFETY/2002/epogen.htm [computer program]. 2002.
94. Combe C, Tredree RL, Schellekens H. Biosimilar epoetins: an analysis based on recently implemented European medicines evaluation agency guidelines on comparability of biopharmaceutical proteins. Pharmacotherapy 2005; 25: 954-62.
95. MacDougall IC. CERA (Continuous Erythropoietin Receptor Activator): a new erythropoiesis-stimulating agent for the treatment of anemia. Curr Hematol Rep 2005; 4: 436-40.
96. Delgado C, Francis GE, Fisher D. The uses and properties of PEG-linked proteins. Crit Rev Ther Drug Carrier Syst 1992; 9: 249-304.
97. Mehvar R. Modulation of the pharmacokinetics and pharmacodynamics of proteins by polyethylene glycol conjugation. J Pharm Pharmacol 2000; 3: 125-36.
98. Osterborg A. New erythropoietic proteins: rationale and clinical data. Semin Oncol 2004; 31: 12-8.
99. Jolling K, Perez Ruixo JJ, Hemeryck A, Vermeulen A, Greway T. Mixed-effects modelling of the interspecies pharmacokinetic scaling of pegylated human erythropoietin. Eur J Pharm Sci 2005; 24: 465-75.
100. Long DL, Doherty DH, Eisenberg SP, Smith DJ, Rosendahl MS, Christensen KR, et al. Design of homogeneous, monopegylated erythropoietin analogs with preserved in vitro bioactivity. Exp Hematol 2006; 34: 697-704.

101. Kochendoerfer GG, Chen SY, Mao F, Cressman S, Traviglia S, Shao H, et al. Design and chemical synthesis of a homogeneous polymer-modified erythropoiesis protein. Science 2003; 299: 884-7.
102. Chen SY, Cressman S, Mao F, Shao H, Low DW, Beilan HS, et al. Synthetic erythropoietic proteins: tuning biological performance by site-specific polymer attachment. Chem Biol 2005; 12: 371-83.
103. Lee DE, Son W, Ha BJ, Oh MS, Yoo OJ. The prolonged half-lives of new erythropoietin derivatives via peptide addition. Biochem Biophys Res Commun 2006; 339: 380-5.
104. Sytkowski AJ, Lunn ED, Risinger MA, Davis KL. An erythropoietin fusion protein comprised of identical repeating domains exhibits enhanced biological properties. J Biol Chem 1999; 274: 24773-8.
105. Dalle B, Henri A, Rouyer-Fessard P, Bettan M, Scherman D, Beuzard Y, et al. Dimeric erythropoietin fusion protein with enhanced erythropoietic activity in vitro and in vivo. Blood 2001; 97: 3776-82.
106. Way JC, Lauder S, Brunkhorst B, Kong SM, Qi A, Webster G, et al. Improvement of Fc-erythropoietin structure and pharmacokinetics by modification at a disulfide bond. Protein Eng Des Sel 2005; 18: 111-8.
107. Dumont JA, Bitonti AJ, Clark D, Evans S, Pickford M, Newman SP. Delivery of an erythropoietin-Fc fusion protein by inhalation in humans through an immunoglobulin transport pathway. J Aerosol Med 2005; 18: 294-303.
108. Mitragotri S, Blankschtein D, Langer R. Ultrasound-mediated transdermal protein delivery. Science 1995; 269: 850-3.
109. Maitani Y, Moriya H, Shimoda N, Takayama K, Nagai T. Distribution characteristics of entrapped recombinant human erythropoietin in liposomes and its intestinal absorption in rats. Int J Pharm 1999; 185: 13-22.
110. Johnson DL, Farrell FX, Barbone FP, McMahon FJ, Tullai J, Kroon D, et al. Amino-terminal dimerization of an erythropoietin mimetic peptide results in increased erythropoietic activity. Chem Biol 1997; 4: 939-50.
111. Fan Q, Leuther KK, Holmes CP, Fong KL, Zhang J, Velkovska S, et al. Preclinical evaluation of Hematide, a novel erythropoiesis stimulating agent, for the treatment of anemia. Exp Hematol 2006; 34: 1303-11.
112. Stead RB, Lambert J, Wessels D, Iwashita JS, Leuther KK, Woodburn KW, et al. Evaluation of the safety and pharmacodynamics of Hematide, a novel erythropoietic agent, in a phase 1, double-blind, placebo-controlled, dose-escalation study in healthy volunteers. Blood 2006; 108: 1830-4.
113. Qureshi SA, Kim RM, Konteatis Z, Biazzo DE, Motamedi H, Rodrigues R, et al. Mimicry of erythropoietin by a nonpeptide molecule. Proc Natl Acad Sci USA 1999; 96: 12156-61.
114. Goldberg J, Jin Q, Ambroise Y, Satoh S, Desharnais J, Capps K, et al. Erythropoietin mimetics derived from solution phase combinatorial libraries. J Am Chem Soc 2002; 124: 544-55.
115. Wang GL, Semenza GL. Desferrioxamine induces erythropoietin gene expression and hypoxia-inducible factor 1 DNA-binding activity: implications for models of hypoxia signal transduction. Blood 1993; 82: 3610-5.
116. Gleadle JM, Ebert BL, Firth JD, Ratcliffe PJ. Regulation of angiogenic growth factor expression by hypoxia, transition metals, and chelating agents. Am J Physiol 1995; 268: C1362-C1368.
117. Wanner RM, Spielmann P, Stroka DM, Camenisch G, Camenisch I, Scheid A, et al. Epolones induce erythropoietin expression via hypoxia-inducible factor-1α activation. Blood 2000; 96: 1558-65.
118. Linden T, Katschinski DM, Eckhardt K, Scheid A, Pagel H, Wenger RH. The antimycotic ciclopirox olamine induces HIF-1alpha stability, VEGF expression, and angiogenesis. FASEB J 2003; 17: 761-3.
119. Kling PJ, Dragsten PR, Roberts RA, Dos-Santos B, Brooks DJ, Hedlund BE, et al. Iron deprivation increases erythropoietin production in vitro, in normal subjects and patients with malignancy. Br J Haematol 1996; 95: 241-8.
120. Ren X, Dorrington KL, Maxwell PH, Robbins PA. Effects of desferrioxamine on serum erythropoietin and ventilatory sensitivity to hypoxia in humans. J Appl Physiol 2000; 89: 680-6.
121. Salnikow K, Su W, Blagosklonny MV, Costa M. Carcinogenic metals induce hypoxia-inducible factor-stimulated transcription by reactive oxygen species-independent mechanism. Cancer Res 2000; 60: 3375-8.
122. Topol IA, Nemukhin AV, Salnikow K, Cachau RE, Abashkin YG, Kasprzak KS, et al. Quantum chemical modeling of reaction mechanism for 2-oxoglutarate dependent enzymes: effect of substitution of iron by nickel and cobalt. J Phys Chem A Mol Spectrosc Kinet Environ Gen Theory 2006; 110: 4223-8.
123. Yuan Y, Hilliard G, Ferguson T, Millhorn DE. Cobalt inhibits the interaction between hypoxia-inducible factor-alpha and von Hippel-Lindau protein by direct binding to hypoxia-inducible factor-alpha. J Biol Chem 2003; 278: 15911-6.
124. Goldwasser E, Jacobson LO, Fried W, Plzak LF. Studies on erythropoiesis. V. The effect of cobalt on the production of erythropoietin. Blood 1958; 13: 55-60.
125. Jelkmann W. Biochemistry and assays of Epo. In: Jelkmann W, ed. Erythropoietin: Molecular Biology and Clinical Use. 1 ed. Johnson City, TN, USA: FP Graham Publishing Co.; 2003: 35-63.
126. Weissbecker L. Die Kobalttherapie. Dtsch Med Wochenschr 1950; 75: 116-8.
127. Lippi G, Franchini M, Guidi GC. Cobalt chloride administration in athletes: a new perspective in blood doping? Br J Sports Med 2005; 39: 872-3.
128. Majamaa K, Hanauske-Abel HM, Gunzler V, Kivirikko KI. The 2-oxoglutarate binding site of prolyl 4-hydroxylase. Identification of distinct subsites and evidence for 2-oxoglutarate decarboxylation in a ligand reaction at the enzyme-bound ferrous ion. Eur J Biochem 1984; 138: 239-45.
129. Tschank G, Raghunath M, Gunzler V, Hanauske-Abel HM. Pyridinedicarboxylates, the first mechanism-derived inhibitors for prolyl 4-hydroxylase, selectively suppress cellular hydroxyprolyl biosynthesis. Decrease in interstitial collagen and Clq secretion in cell culture. Biochem J 1987; 248: 625-33.
130. Franklin TJ, Morris WP, Edwards PN, Large MS, Stephenson R. Inhibition of prolyl 4-hydroxylase in vitro and in vivo by members of a novel series of phenanthrolinones. Biochem J 2001; 353: 333-8.
131. Kivirikko KI, Myllyla R, Pihlajaniemi T. Protein hydroxylation: prolyl 4-hydroxylase, an enzyme with four cosubstrates and a multifunctional subunit. FASEB J 1989; 3: 1609-17.
132. Baader E, Tschank G, Baringhaus KH, Burghard H, Gunzler V. Inhibition of prolyl 4-hydroxylase by oxalyl amino acid derivatives in vitro, in isolated microsomes and in embryonic chicken tissues. Biochem J 1994; 300: 525-30.
133. Safran M, Kim WY, O'Connell F, Flippin L, Gunzler V, Horner JW, et al. Mouse model for noninvasive imaging of HIF prolyl hydroxylase activity: assessment of an oral agent that stimulates erythropoietin production. Proc Natl Acad Sci U S A 2006; 103: 105-10.
134. Urquilla P, Fong A, Oksanen S, Leigh S, Turtle E, Flippin L, et al. Upregulation of endogenous EPO in healthy sbjects by inhibition of HIF-PH. J Am Soc Nephrol 2004; 15: 546.
135. Wiecek A, Piecha G, Ignacy W, Schmidt R, Neumayer HH, Scigalla P, et al. Pharmacological stabilisation of HIF increases hemoglobin concentration in anemic patients with chronic kidney disease. Nephrol Dial Transplant 2005; 20 (Suppl. 5): 195.
136. Duntas LH, Parisis C. Doping: a challenge to the endocrinologist. A reappraisal in view of the Olympic Games of 2004. Hormones 2003; 2: 35-42.

137. Imagawa S, Nakano Y, Obara N, Suzuki N, Doi T, Kodama T, et al. A GATA-specific inhibitor (K-7174) rescues anemia induced by IL-1beta, TNF-alpha, or L-NMMA. FASEB J 2003; 17: 1742-4.
138. Nakano Y, Imagawa S, Matsumoto K, Stockmann C, Obara N, Suzuki N, et al. Oral administration of K-11706 inhibits GATA binding activity, enhances hypoxia-inducible factor 1 binding activity, and restores indicators in an in vivo mouse model of anemia of chronic disease. Blood 2004; 104: 4300-7.
139. Barbone FP, Johnson DL, Farrell FX, Collins A, Middleton SA, McMahon FJ, et al. New epoetin molecules and novel therapeutic approaches. Nephrol Dial Transplant 1999; 14: 80-4.
140. Jelkmann W. Erythropoietin after a century of research: younger than ever. Eur J Haematol 2007; 78: 183-205.

Received: April 18, 2007
Accepted: April 26, 2007
Published: April 27, 2007

Address for correspondence:
Wolfgang Jelkmann, M.D.
Institute of Physiology
University of Luebeck
Ratzeburger Allee 160
D – 23538 Luebeck, Germany
jelkmann@physio.uni-luebeck.de

Author's contribution
A – Study Design
B – Data Collection
C – Statistical Analysis
D – Data Interpretation
E – Manuscript Preparation
F – Literature Search
G – Funds Collection

Medicina Sportiva, Vol. 11 (2): 43-47, 2007
DOI: 10.2478/v10036-007-0008-0

THE INFLUENCE OF SHORT-TERM MAXIMAL ANAEROBIC AND AEROBIC EXERTION ON AUTONOMIC NERVOUS SYSTEM ACTIVITY IN HEALTHY YOUNG ATHLETES

Ludmiła Daniłowicz-Szymanowicz[1 (A, B, C, D, F, F, G)], Grzegorz Raczak[1(A, D, F)], Małgorzata Szwoch[1(B, E)], Wojciech Ratkowski[2(B,)], Antoni Bogdan Toruński[1(B)]

[1]II Department of Cardiology, Medical University of Gdańsk, Poland
[2]Academy of Physical Education and Sport in Gdańsk, Poland

Abstract

Daniłowicz L, Raczak G, Szwoch M, Ratkowski W, Toruński AB. The influence of short-therm maximal anaerobic and aerobic exertion on autonomic nervous system activity in healthy young athletes. *Med Sport* 2007; 11(2): 43-47.

Introduction: The Wingate test (WT) and incremental test for maximal oxygen uptake (IT) are used in the evaluation of physical efficiency in professional athletes. In the former anaerobic power is evaluated and in the latter aerobic power.

Aim: The aim of the study was to evaluate the effect of the tests on autonomic nervous system activity (ANS) in young healthy athletes.

Material and methods: Eight athletes aged 17± 1 were included in the study. On the first day WT was performed, on the second day the IT. The ANS parameters (baroreflex sensitivity - BRS_WBA and heart rate variability - HRV) were analyzed on the basis of 10-minute systolic arterial pressure (SAP) and heart period (HP) records during controlled breathing (0.23 Hz). ANS indices and mean HP were analyzed before (examination 1) and within 1 hour after WT (examination 2), and within 1 hour after IT (examination 3).

Results: The significant decrease in BRS-WBA in examination 2 in comparison to 1 was found (18.1±10.7 vs. 9.4±3.9 ms/mmHg, p=0.049). In examination 3 in comparison to 1 the significant decrease in BRS_WBA was also found (7.7±5.2 ms/mmHg, p=0.011). SDNN, PNN50, RMSSD, HF and HFnu were significantly lower in examination 2 comparing to 1 (p<0.05). These lower values were noticed also after examination 3. The mean HP showed similar changes. LF/HF and LFnu increased significantly in examination 2 in comparison to 1 (p<0.05). LF did not change significantly during the examinations.

Conclusions: Short-term maximal complex exertion during anaerobic and aerobic tests leads to the decrease in parasympathetic activity and to the increase in sympathetic activity of ANS in young healthy athletes. These changes last at least one hour after exertion.

Key words: *autonomic nervous system, baroreflex sensitivity, physical exercise*

Introduction

Two tests that are commonly used for evaluation of athletes' preparation for competition are the Wingate test and incremental test for maximal oxygen uptake. The former test is used to evaluate anaerobic power while the latter is used to test aerobic power. The influence of the complex exertion in these two tests on autonomic nervous system (ANS) activity is not fully examined. From the practical point of view, it seems important to investigate this issue.

The problem described above is a result of the fact that the influence of exertion on cardiovascular system depends on various factors, including intensity, type and duration of the exercise. Moderate single exertion applied as a recreation activity in sedentary people may lead to increased parasympathetic activity of ANS (1). Physical activity lasting several months in these people, as well as long-lasting intensive training in group of athletes preparing for competitions, lead to beneficial changes in sympathetic-parasympathetic balance (2,3). However, high increase in exertion intensity may result in adverse permanent shift of ANS activity from parasympathetic domination to sympathetic one, as it was demonstrated by Iellamo et al in his study of Italian rowing champions (4). Persistent sympathetic activation was also reported as a result of short-term exertion such as marathon race or strength-testing competition (5-7).

Available reports on the influence of combination of Wingate test and incremental test for maximal oxygen uptake on ANS activity provide ambiguous data. Complex nature of influence of physical exercise on ANS makes it impossible to predict the sympathetic-parasympathetic balance changes caused by these two tests. On the other hand, the exertion applied to professional athletes preparing for competition, undergoing these tests, is very intensive. Therefore, it cannot be assumed that the impact on ANS function is not important.

It is important to note that spontaneous breathing by the examined person has a significant influence on

ANS activity. For example, breathing at the rate of 15 breaths per minute is equal to 0.25 Hz, but reduction of breath rate to 6 breaths per minute (0.1 Hz) may influence ANS indices in low frequency band (0.04-0.15 Hz). This fact is particularly important in a group of professional athletes who tend to breathe at a lower rate than sedentary people.

Aim

The aim of the study was to assess the influence of short-term, extreme exertion during Wingate test and incremental test for maximal oxygen uptake on the ANS function in professional athletes preparing for the competition.

Material and Methods

Eight young healthy athletes aged 17 ± 1 (16-19 year), preparing for the competition, were included in the study. Additional inclusion criteria were: absence of any disease (particularly ones of cardiovascular origin), no drugs and alcohol intake, no cigarette smoking, sinus rhythm in ECG and consent to participate in the study

According to the study protocol, each person was examined three times in the morning: 1) before the Wingate test, 2) within one-hour period after the Wingate test, 3) on the following day, within one-hour period after the incremental test for maximal oxygen uptake. Examinees did not eat for at least four hours and did not smoke cigarettes or drink coffee for twelve hours before the tests. In each examined person, a ten-minute recording of systolic arterial pressure (SAP) and heart period (HP) were performed. Tests were carried out in full compliance with the detailed protocol, in a quiet laboratory room, while the examined persons were relaxed and lying in supine position, with head lifted by 30°. During the first 15 minutes of each test, recordings were not made, because the stabilisation of SAP and HP took place. After that, the SAP and HP were recorded during paced breathing (RC) at a rate of 0.23 Hz, synchronized by voice played back from tape. The obtained SAP and HP recordings were used for analysis of baroreflex sensitivity (BRS), heart rate variability (HRV) and mean heart period (mean HP).

Tests of physical efficiency

Tests of physical efficiency were performed in the laboratory of Academy of Physical Education and Sport in Gdańsk. The Wingate test was performed on the first day and the incremental test for maximum oxygen uptake was performed on the following day.

Wingate test

The anaerobic efficiency was assessed in athletes using the 30-second version of the Wingate test. Each test started with a 5-minute warm-up using the cycloergometer, with maximum intensity of exertion defined by heart rate of 140-150/min. After a 5-minute rest, the main test was started. The athletes aimed at achieving maximum rate of pedalling in the shortest possible time and maintaining this rate as long as they could. In this study, cycloergometer Monark 824-E with mechanically adjusted resistance of the flywheel was used. The parameters assessing the anaerobic efficiency (the maximum power, time needed for achieving and maintaining it and the amount of work done) were calculated using MCE V2.0 computer software.

The incremental test for maximum oxygen uptake

The incremental test for maximum oxygen uptake, was performed using the cycloergometer in order to assess the aerobic efficiency in the athletes. The examinees performed the test with gradually increased load, until the maximum oxygen uptake was obtained. After a 2-minute recording made at rest, the athletes were pedalling without the load for 3 minutes, maintaining the pedalling rate of 50/min. For the next 5 minutes, the examinees were pedalling with load of 100 W, maintaining the rate of 50/min. From the 10th minute of the test, the load was increased by 25W every one minute. The exertion was continued until the test was stopped or when the pedalling rate decreased below 10% of the required value of 50/min.

The telemetric exhaled gases analyser (made by Cosmed) was used to analyse aerobic power indices: $\dot{V}O_2max$ – maximum oxygen uptake, and AT – aerobic-anaerobic threshold. During the tests, oxygen uptake (VO_2), carbon dioxide exhaling (VCO_2) and minute ventilation (VE) were monitored continuously. Heart rate was measured using the Polar sport tester, synchronised with the exhaled gases analyser. The AT was calculated using the indirect method, from VE analysis results (during the rapid occurrence of non-linear VE recording in the test). Respiratory quotient (RQ) was also used to assess AT. It was assumed that AT threshold is achieved when RQ is equal to or above 1.

Assessment of ANS activity

Signal of SAP was obtained using non-invasive beat-to-beat method using FINAPRES (Ohmeda), with sleeve placed on middle phalanx of the third digit of the right hand. HP recording was performed using MINGOGRAF 720C. Self-adjustment FINAPRES function was switched off directly before the actual recording and it was switched on again after each recording in order to recalibrate the device.

The recorded analog SAP and HP signals were synchronised and processed by analog-to-digital converter with sampling frequency of 250 Hz and transferred to computer with POLYAN software (8)

that calculated BRS and HRV indices. HP signal resolution equal to 1 ms was obtained using linear interpolation algorithm.

The assessment of BRS indices was preceded by removal of the ectopic beats and trends from the recordings. Next, a fragment of stable SAP and HP recording, not shorter than 240 seconds, was selected for the analysis. BRS was evaluated automatically, which decreased the subjectivity of the analysis. Value of BRS_WBA was calculated from spectral analysis of spontaneous SAP and HP variations using Blackman-Tukey algorithm with Parzen window of 0.03 Hz bandwidth, as an averaged module of transfer function in the 0.04-0.15 Hz range, using all points of SAP and HP function regardless of coherence value and variability (9). Values of BRS_WBA were expressed in ms/mmHg.

The following parameters were included in analysis of short-term HRV (10):

SDNN (ms) – standard deviation of RR intervals over the selected time interval

pNN50 (%) – percentage of intervals differing by more than 50 ms from the preceding interval

RMSSD (ms) – root-mean-square value of differences between adjacent RR intervals

TP (ms^2) – total spectral power (ms^2)

LF (ms^2) – power of low frequency components (0.04-0.15 Hz)

HP (ms^2) – power of high frequency components (0.15-0.4 Hz)

LF_{nu} – normalized LF power

HF_{nu} – normalized HF power

LF/HF – ratio of LF power to HF power

The tests were an integral part of a larger program for assessment of influence of physical exercise on ANS function. A consent to perform the study was received from the Independent Bioethical Committee for Scientific Research in Medical University of Gdańsk (NKEBN/10/2003).

Statistical analysis

Statistica 6.0 computer software was used for statistical analysis of the data. Continuous variables were expressed as mean ± standard deviation (SD). The significance of differences between the indices in the subsequent recordings with reference to basal values was assessed using the matched-pair Wilcoxon test, because the distribution of variables was not normal. The value of $P \leq 0.05$ was assumed to be statistically significant.

Results

Maximum oxygen uptake ($\dot{V}O_2max$) in the examined athletes was equal to 53.6 ± 6.0 ml/kg/min. In all examined persons, diagnostic values of ANS parameters were obtained.

Barorefl;ex sensitivity was decreased after the Wingate test comparing to the initial values: from 18.1 ± 10.7 to 9.4 ± 3.9 ms/mmHg (p= 0.049). After incremental test for maximum oxygen uptake, the value of BRS_WBA also decreased comparing to the first test (7.7 ± 5.2 ms/mmHg, p= 0.011). These results are illustrated in Figure 1.

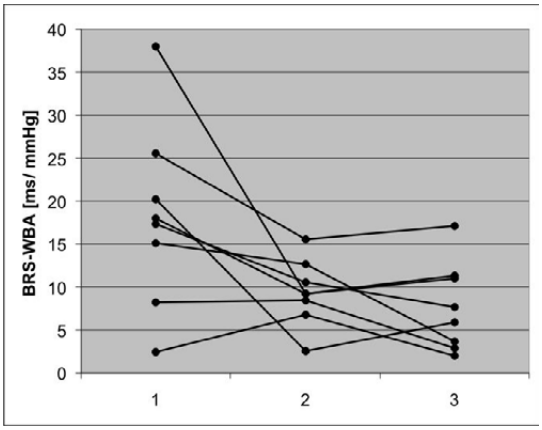

Figure 1. *BRS_WBA before (1) and within 1 hour after Wingate test (2), within 1 hour after incremental test for maximal oxygen uptake (3)*
BRS_WBA (1) vs BRA_WBA (2)- p=0.049
BRS_WBA (1) vs BRS_WBA (3)- p=0.011

Table 1 presents data concerning HRV parameters and mean HP values obtained in the performed tests. It is important to note that all time-domain HRV indices were significantly decreased after the Wingate test, comparing to their initial values. The decreased values of SDNN and pNN50 persisted after the incremental test for maximum oxygen uptake. Similar trends were observed in case of mean HP. The increase of RMSSD value after incremental test for maximum oxygen uptake was observed. However, this change was statistically insignificant comparing to the previous recording.

Table 1. *HRV indices and mean HP before (1) and within 1 hour after Wingate test (2), within 1 hour after incremental test for maximal oxygen uptake (3)*

	1	2	3
SDNN	91 ± 41*♦	65 ± 26	64 ± 38
PNN50	56 ± 25*♦	36 ± 20	36 ± 19
RMSSD	108 ± 68*♦	62 ± 28	75 ± 52
TP	6927 ± 7725♦	4448 ± 3188	4589 ± 7283
LF	1215 ± 813	1421 ± 1307	1098 ± 1277
Fnu	20 ± 13*♦	35 ± 16	35 ± 14
HF	7312 ± 8038*♦	2458 ± 1984	3383 ± 5706
HFnu	80 ± 13*♦	65 ± 16	65 ± 14
LF/ HF	0,29 ± 0,24*♦	0,61 ± 0,39	0,60 ± 0,36
Mean HP	944 ± 111*	843 ± 57	893 ± 43

* 1 vs 2 p<0.05; ♦ 1 vs 3 p<0.05; ♦♦ 2 vs 3 p<0.05

In spectral analysis, changes of TP, HF and HF_{nu} were similar to those observed in time-domain. In the second recording, statistically significant decrease of HF and HF_{nu} values. The lower values of TP and HF_{nu} were also noticed in the third recording. Increase of HF value in comparison to the second recording was found, but it was statistically insignificant.

In analysis of LF_{nu} and LF/HF parameters, statistically significant increase was observed in the second recording. After the incremental test for maximum oxygen uptake, values of these parameters were also significantly higher comparing to the first recording. LF value did not change significantly during the tests.

Discussion

The main observation in this study is that short-term, complex anaerobic-aerobic maximum exertion in professional athletes results in decrease of parasympathetic component of ANS and increase of sympathetic component of ANS. These changes are observed after the Wingate test and they persist after the incremental test for maximum oxygen uptake.

The data in the literature, suggesting the favourable increase of parasympathetic activity after a single exercise, concern an exertion of moderate intensity. For example, Pober et al observed in a group of young sedentary volunteers, the increase of HF and pNN50 and decrease of LF and LF/HF, as a result of 60-minute cycling with intensity needed to achieve 65% of maximum oxygen uptake (11). The previous reports published by the authors showed a significant increase of SDNN and TF-BRS values after 30-minute running on a treadmill, performed until heart rate was equal to 130/min, in a similar group of young people (1). However, the exertion applied in the mentioned studies was less intensive than during anaerobic and aerobic tests in professional athletes examined in this study.

An adverse, from the clinical point of view, reduction of ANS parasympathetic activity and increase of sympathetic one after the extreme exertion after the one-time, extreme exertion was reported by several researchers (5-7,12). For example, Bernardi et al evaluated the influence of a 46-kilometer mountain marathon and they have shown the decrease of SDNN and HF and the increase of LF/HF (5). In recent studies published by the authors, the decrease of BRS-WBA and increase of LF/HF value after a 42-kilometer marathon were reported (6). Similar changes were observed in the study by Gratze et al, assessing athletes taking part in strength-testing competition (7). In this study, increase of parasympathetic activity was obtained and it persisted for at least 60 minutes after each test, although the exertion was applied for much shorter time, comparing to the studies mentioned above. It should also be noted that increased sympathetic activity as a response to anaerobic-aerobic exertion, found in this study, is of particular importance, because of exclusion of spontaneous breathing that could influence the indices measured in low frequency range (0.04-0.15 Hz) in examined athletes. It was achieved by imposing the paced breathing at a rate of 0.23 Hz.

On the other hand, it should be stressed that although the maximum exertion was applied in athletes examined in anaerobic and aerobic tests, no further adverse increase in sympathetic activity was observed after the incremental test for maximum oxygen uptake, performed on the following day, comparing to results obtained on the first day after the Wingate test, for all parameters assessed in this study. Therefore, it may be assumed that the protocol of anaerobic and aerobic tests used in the study is safe for the examined athletes.

Conclusions

Short-term, complex, maximum exertion in anaerobic and aerobic tests, performed in professional athletes in order to evaluate their level of preparation for competition, results in decreased ANS parasympathetic activity and increased sympathetic one. These changes persist for at least one hour after exertion.

References

1. Raczak G, Pinna GD, La Rovere MT, Maestri R., et al. Cardiovascular Response to Acute Mild Exercise in Young Healthy Subjects. *Circ J* 2005; 69: 976- 80.
2. Hedelin R, Wiklund U, Bjerle P, Henriksson- Larsen K. Pre- and post- season heart rate variability on adolescent cross- country skiers. *Scand J Med Sci Sports* 2000; 10: 298- 303.
3. Raczak G, Daniłowicz-Szymanowicz L, Kobuszewska-Chwirot M, Ratkowski W., et al. Long-term exercise training improves autonomic nervous activity profile in professional runners. *Kardiol Pol* 2006; 64: 135-40.
4. Iellamo F, Legramante JM, Pigozzi F, Spataro A., et al. Conversion from vagal to sympathetic predominance with strenuous training in high- performance world class athletes. *Circulation* 2000; 105: 2719- 24.
5. Bernardi L, Passino C, Robergs R, Appenzeller O. Acute and persistent effects of a 46-kilometer wilderness trail run at altitude: cardiovascular autonomic modulation and baroreflexes. *Cardiovasc Res* 1997; 34: 273- 80.
6. Daniłowicz-Szymanowicz L, Raczak G, Pinna GD, Maestri R., et al. Wpływ jednorazowego ekstremalnego wysiłku fizycznego o charakterze wytrzymałościowym na czynność autonomicznego układu nerwowego. *Pol Merk Lek* 2005; 109: 28- 31.
7. Gratze G, Rudnicki R, Urban W, Mayer H., et al. Hemodynamic and autonomic changes induced by Ironman: prediction of competition time by blood pressure variability. *J Appl Physiol* 2005; 99: 1728- 35.
8. Maestri R, Pinna GD. POLYAN: a computer program for polyparametric analysis of cardio- respiratory variability signals. *Comput Methods Programs Biomed* 1998; 56: 37- 48.
9. Pinna GD, Maestri R, Raczak G, La Rovere MT. Measuring baroreflex sensitivity from gain function between arterial pressure and heart period. *Clin Sci* 2002; 103: 81- 8.
10. Camm AJ, Bigger JT, Breithardt G, Cohen RJ., et al. Heart rate variability. Standards of measurement, physiological interpretation, and clinical use. *Eur Heart J* 1996; 17: 354-81.

11. Pober DM, Braun B, Freedson PS. Effects of a single bout of exercise on resting heart rate variability. *Med Sci Sports Exerc* 2004; 36: 1140-8.
12. Heffernan KS, Kelly EE, Collier SR, Fernhall B. Cardiac autonomic modulation during recovery from acute endurance versus resistance exercise. *Eur J Cardiovasc Prev Rehabil* 2006; 13: 80- 6.

Received: July 01, 2006
Accepted: May 31, 2007
Published: June 27, 2007

Address for correspondence:
Dr Ludmiła Daniłowicz-Szymanowicz
ul. Dębinki 7
80- 952 Gdańsk
Poland
tel. (+48) 600-101-106
e- mail: ludwik@amg.gda.pl

Author's contribution
A – Study Design
B – Data Collection
C – Statistical Analysis
D – Data Interpretation
E – Manuscript Preparation
F – Literature Search
G – Funds Collection

Medicina Sportiva, Vol. 11 (2): 48-50, 2007
DOI: 10.2478/v10036-007-0009-z

INFLUENCE OF PROLONGED EXERCISE ON THE 24-HOUR FREE TESTOSTERONE – CORTISOL RATIO HORMONAL PROFILE

Joseph W. Duke [D,E], Daniela A. Rubin [B], Will Daly [B], Anthony C. Hackney [A,B,D,E]

Endocrine Section – Applied Physiology Laboratory, Department of Exercise & Sport Science, University of North Carolina, Chapel Hill, North Carolina, USA

Abstract

Duke JW, Rubin DA, Daly W, Hackney AC. Influence of prolonged exercise on the 24-hour free testosterone – cortisol ratio hormonal profile. *Med Sport* 2007, 11(2):48-50.

Aim of the study: This study examined the response of the free testosterone and cortisol ratio (fTC) to prolonged endurance exercise.

Methods: A trained sportsman performed treadmill exercise at 70% of maximal oxygen uptake to volitional fatigue. Frequent blood samples were collected over 24 hours and analyzed to determine changes in the fTC ratio.

Results: Exercise caused an initial increase in the ratio followed by a rapid decline in the immediate recovery from exercise. Approximately 8 hours into recovery from exercise, there was a pronounced and persistent (~6 hours) secondary increase in the ratio.

Conclusions: Results suggest that the fTC ratio responds to intensive exercise, but that aspects of the response can be of a delayed nature occurring many hours into recovery. Exercise scientists who wish to monitor this parameter should be aware that the time course by which they choose to conduct their blood sampling could impact upon the interpretation of their data.

Key words: *endocrine, stress, training biomarkers*

Introduction

Athletes participating in sporting events subject their bodies to an extreme amount of stress in performing their daily training and sports competitions. This stress is necessary, to a certain degree, in order to facilitate the adaptation process that allows an improvement in human performance (1). When the human body is subjected to stressful events the neuroendocrine system can respond and release a multitude of hormones, which can aid the body in the adaptation process (2).

Many exercise research studies have been conducted to examine the hormonal response to an exercise bout. These studies have provided valuable information on the behavior of the neuroendocrine system. Unfortunately, many of these studies suffer from a major methodological limitation: hormonal assessments based upon an inadequate sampling frequency. This is especially true for studies involving blood sampling. In an attempt to circumvent this problem, less invasive specimens (i.e., urine and saliva) have been obtained. However, these specimens do not allow for as complete a hormonal evaluation as blood specimens (3).

Over 20 years ago, the free testosterone and cortisol (fTC) ratio was proposed as a critical hormonal marker of the anabolic and catabolic status in sportsman (4). Contemporary research suggests the ratio has certain limitations and drawbacks for usage in such a purpose (3,5). Nevertheless, it has remained a useful and popular tool for the monitoring of training stress, especially relative to the development of the Overtraining Syndrome (6,7).

With the above information in mind, our laboratory was presented with what we considered a unique opportunity; i.e., to perform a 24-hour hormonal assessment in an endurance-trained athlete who performed an intensive exercise session that mimicked an intense, prolonged, endurance exercise session. Our purpose in conducting such an evaluation was to provide "reference findings" that might serve as a comparative "normal" fTC ratio hormonal response to such exercise.

Materials and Methods

Hormonal levels were measured in a healthy physically active male (age = 22, height 176 cm, body mass = 72.2kg) who had been involved with exercise training and competitive sport (e.g., running, cycling) for a number of years. His maximal aerobic capacity ($\dot{V}O_2max \approx 60$ ml/kg/min) rated him as being highly trained (8). In accord with the Declaration of Helsinki he approved and signed a written informed consent prior to voluntary participation in the study.

Serial blood specimens were collected from the subject for a 24-hour period, following a 12 hr fast. For the day prior to the experiment he performed no physical activity, and for the 3 days before the experiment, he followed a controlled diet to ensure adequate carbohydrate and total caloric intakes were maintained.

On the day of the experiment he reported to our laboratory in the morning and an antecubial venous catheter was inserted into his right arm. The catheter was kept patent with a saline infusion solution. The subject next rested quietly for 30 minutes supine, and then an initial baseline blood specimen was taken, followed by a 5 minute active warm-up period. The subject then began running on a motorized treadmill at an intensity corresponding to ~70% of his VO_{2max} until he reached the point of volitional fatigue. During exercise, blood specimens were taken at 15-minute intervals and additional specimens were obtained at 0.5, 1, 1.5, 2 hours into recovery. Subsequent to this sampling, blood specimens were collected on an hourly basis until a 24-hour period was reached. During recovery the subject remained in the laboratory and rested quietly (supine - reading, television watching) or slept, and consumed water freely as well as evening (see Figure 1, 0800 hours) and breakfast meals (2100 hours; 2500 kcal total intake).

The blood samples were all treated appropriately to insure viable hormonal analysis (specific details are reported elsewhere; 9). Briefly, hormonal concentrations were measured in duplicate and were analyzed with an ultra-sensitive, single-ligand, solid phase methodology radioimmunoassay technique (DPC Inc., Los Angles, CA, USA). Hormones measured were free testosterone as an anabolic index and cortisol as a catabolic index. Additionally, sex hormone binding globulin (SHBG) was measured only in the initial and final resting blood samples. Specific assay sensitivities were; 4.0 pmol·L^{-1}, 5.5 nmol·L^{-1}, and 0.04 nmol·L^{-1} for free testosterone, cortisol, and SHBG, respectively. The between and within assay coefficients of variation for all assays were ≤8.2% and ≤6.6%, respectively.

Results and Discussion

The subject exercised for a total of 82.2 minutes before he reached volitional fatigue and stopped. The fTC results are depicted in Figure 1. The figure clearly indicates that the exercise caused an initial, substantial rise in the ratio, but was followed by a large decline that continued into early recovery. Most surprisingly was the finding that at approximately 8 hours into recovery there was another substantial and persistent elevation in the ratio (lasting approximately 6 hours). This was followed by another large reduction and a minor elevation during the period of sleep. The hormonal changes far exceeded what would be expected for plasma volume induced changes due to fluids shifts during the exercise (2). The SHBG concentrations in the initial and final blood sample were 36.5 nmol·L^{-1} and 38.3 nmol·L^{-1}, respectfully. These, as well as, the hormonal values were all within normal clinical reference ranges for physically active males (10).

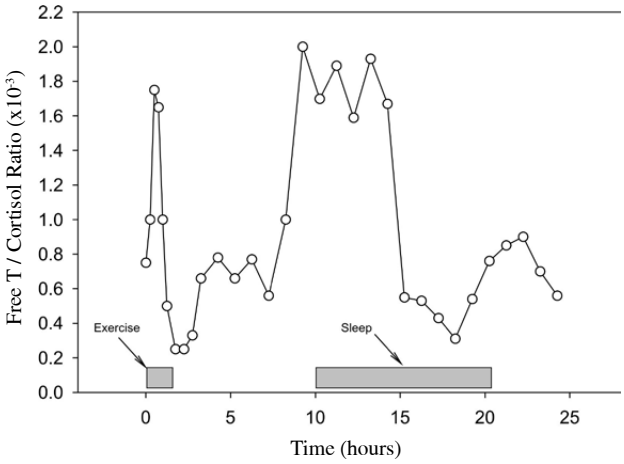

Fig 1. *Free testosterone and cortisol ratio (fTC) versus time during the 24 hour period following the prolonged endurance event. The "0" time point refers to the sample taken immediately pre-exercise.*

It is realized that these data are limited due to the case study nature of the study. Nonetheless, the results may prove to be useful to exercise scientists working in the endocrinology area for several reasons. First, the findings suggest that judgments on the effect of exercise to influence the fTC ratio may be dramatically affected by when the timing of the blood sampling occurs following the exercise session. Secondly, relative to the anabolic processes critical to training adaptation (i.e., muscle tissue repair) in the body, the findings suggest it may be essential that the nutritional substrates needed for such hormonal mediated processes be available at the beginning of the delayed elevation in the fTC ratio approximately 8 hours into recovery (2). Finally, from a clinical perspective these results would indicate that certain aspects of the hormonal profiles of an athletic individual, on a day in which they perform intensive exercise, will vary from what is typically considered as a normal hormonal profile. Specifically, at certain times the hormonal levels will be far greater or far lower than the expected range of values as reported in clinical reference textbooks (10).

Another potential limitation to the study involves the methodological aspects of the free testosterone assay which was utilized. Vermeulen et al. (11) has argued that this immunological procedure is not precise enough. This is a legitimate concern, but it is important to point out that the present changes observed in the free testosterone concentrations were substantially large and not of a minute level where precision could

have been a critical factor. Furthermore, in the present study select SHBG levels (research funding considerations prevented further analysis) were measured and a free level of testosterone was calculated using the procedures of Nanjee and Wheeler (12). The calculation-derived values were very similar to what had been measured within our assay. Collectively, these points are suggestive that our testosterone data do accurately reflect the physiological status of our subject.

In conclusion, the findings from this case study suggest that the fTC ratio responds to intensive exercise, but that aspects of the response can be of a delayed nature occurring many hours into recovery. Furthermore, scientists monitoring the fTC should be conscious that the time course during which they choose to conduct blood sampling could impact upon the interpretation of their data.

References

1. Viru A, Viru M. Cortisol – essential adaptation hormone in exercise. *Int J Sports Med* 2004; 25: 461-4.
2. McMurray RG, Hackney AC. Endocrine responses to exercise and training. In: Garrett WE, Kirkendall DT., eds. Exercise and Sport Science. Philadelphia, Lippincott Williams & Wilkins 2000, 135-56.
3. Viru A, Viru M. Biochemical Monitoring of Sport and Training. Champaign: Human Kinetics, 2001.
4. Aldercreutz H, Harkonen M, Kuoppasalmi K, et al. Effect of training on plasma anabolic and catabolic steroid hormones and their response during physical exercise. *Int J Sports Med* 1986; 7(suppl.): 27-8.
5. Fry AC, Steinacker JM, Meeusen R. Endocrinology of overtraining. In: Kraaemer WJ, Rogol AD., eds. The Endocrine System in Sports and Exercise. Oxford, UK: Blackwell Publishing 2005: 584-93.
6. Hackney AC. Stress and the neuroendocrine system: the role of exercise as a stressor and modifier of stress. *Expert Reviews in Endocrinology & Metabolism* 2006; 1(6): 783-92.
7. Urhausen A, Gabriel H, Kindermann W. Blood hormones as markers for training stress and overtraining. *Sports Med* 1995; 20(4): 251-76.
8. Shvartz E, Reibold RC. Aerobic fitness norms for males and females aged 6 to 75 years: a review. *Aviat Space Environ Med* 1990; 61(1): 3-11.
9. Daly W, Seegers CA, Rubin DA., et al. Relationship between stress hormones and testosterone with prolonged endurance exercise. *Eur J Appl Physiol* 2005; 93: 375-80.
10. Tietz N. Clinical Guide to Laboratory Tests. London: WB Saunders, 1990.
11. Vermeulen A, Verdonck L, Kaufman JM. A critical evaluation of simple methods for the estimation of free testosterone in serum. *J Clin Endocrinol Metab* 1999; 84(10): 3666-72.
12. Nanjee MN, Wheeler MJ. Plasma free testosterone - is an index sufficient? *Ann Clin Biochem* 1985; 22: 387-90.

Received: March 06, 2007
Accepted : June 29, 2007
Published: June 30, 2007

Address for correspondence
Dr. A.C. Hackney
Applied Physiology Laboratory
University of North Carolina
Chapel Hill, North Carolina
USA
e-mail: ach@email.unc.edu

Author's contribution
A – Study Design
B – Data Collection
C – Statistical Analysis
D – Data Interpretation
E – Manuscript Preparation
F – Literature Search
G – Funds Collection

Medicina Sportiva, Vol. 11 (2): 51-55, 2007
DOI: 10.2478/v10036-007-0010-6

THE EFFECT OF TAI CHI CHUAN TRAINING ON SELECTED METABOLIC PARAMETERS IN ELDERLY MEN

Joanna Karolkiewicz[1(A,B,D,E,F,G)], Łucja Pilaczyńska-Szcześniak[1(A,E,G)], Ewa Deskur-Śmielecka[3(D,E)], Janusz Maciaszek[2(B,G)], Rafał Stemplewski[2(B,G)], Wiesław Osiński[2(B,G)]

[1] Chair of Physiology, Biochemistry and Hygiene, University School of Physical Education, Poznań, Poland
[2] Chair of Theory of Physical Education, University School of Physical Education, Poznań, Poland
[3] Department of Cardiac Rehabilitation, University School of Physical Education, Poznań, Poland

Abstract

Karolkiewicz J, Pilaczyńska-Szcześniak Ł, Deskur-Śmielecka E, Maciaszek J, Stemplewski R, Osiński W. The effect of Tai Chi Chuan training on selected metabolic parameters in elderly men. *Med Sport* 2007, 11(2): 51-55.

The aim of the study was to assess the influence of a 4-month physical training based on Tai Chi exercises on insulin resistance parameters, blood lipids, oxidative stress markers and antioxidant parameters in healthy, elderly men.

Methods: The study population consisted of 41 healthy men, aged 63-75, who volunteered for the training program. The training program lasted 4 months and consisted of two 45-minute sessions of Tai Chi Chuan prophylactic-therapeutic exercises weekly. Total antioxidative status (TAS) and concentrations of thiobarbituric acid reactive substances (TBARS) were measured in plasma. In the serum samples, levels of glucose, HDL-cholesterol, triglycerides and insulin were assessed. Atherogenic index of plasma (AIP) and insulin resistance index ($HOMA_{IR}$) were calculated. Reduced glutathione (GSH) concentrations and glutathione peroxidase activity (GPx) were determined in the red blood cells hemolysate.

Results: The 4-month training based on Tai Chi Chuan exercises resulted in a significant decrease in serum concentrations of glucose, insulin and triglycerides, and a significant increase in HDL-cholesterol levels. $HOMA_{IR}$, TG/HDL-C ratio, and AIP decreased significantly ($p<0.01$). Glutathione peroxidase activity and TBARS concentrations increased significantly ($p<0.01$). No significant changes in concentrations of GSH and TAS were found.

Conclusions: The obtained results show that 4-month training based on Tai Chi Chuan exercises enhances insulin sensitivity, improves lipid profile and the balance between oxidants and antioxidants in healthy, elderly men. These favorable changes may decrease the risk of atherosclerosis in physically active elderly subjects.

Key words: *insulin resistance, antioxidant defense system, blood lipids, physical activity, elderly men*

Introduction

Insulin resistance is defined as a decreased response of peripheral tissues, especially muscular tissue, to insulin action (1). It has been documented that sensitivity to insulin declines with age, at least up to the 9th decade (2). Gumbiner et al. (3) have found that age-related insulin resistance results both from receptor and post-receptor defect. Insulin resistance has been recognized to play a crucial role in the development of several disorders in aging, such as atherosclerosis, hyperglycemia, dyslipidemia, obesity and hypertension (4). It is supposed that good glucose tolerance and high insulin sensitivity favor longevity (5-6). In recent years, the results of numerous studies conducted in elderly and obese populations have linked insulin resistance to systemic oxidative stress (6-8). Oxidative stress impairs glucose uptake in muscle and fat under hyperglycemic condition (4), and decreases insulin secretion from pancreatic beta cells (9). Increased oxidative stress also underlies the pathophysiology of atherosclerosis (10) by directly affecting vascular wall cells.

Increased antioxidant potential may attenuate oxidative stress and thus, presumably, may prevent the development of several age-related disorders and favor successful aging (11). It has been documented that systematic, aerobic physical activity decreases hyperglycemia and hyperinsulinemia, and enhances body antioxidant defense system both in healthy subjects and in obese patients (12-15).

The aim of the study was to assess the effect of a 4-month aerobic training based on Tai Chi Chuan exercises, on insulin resistance parameters, blood lipids, oxidative stress markers and antioxidant parameters in healthy, elderly men.

Material and Methods

The study population consisted of 41 healthy men aged 63–75 years, members of a Senior Club in Poznań, who volunteered for the training program. All subjects declared good health status. Data concerning detailed health condition, dietary habits, alcohol consumption, smoking status and medication were obtained with a questionnaire. Patients with inflam-

matory disorders, recent infections, renal or hepatic insufficiency, active coronary artery disease, diabetes, hypertension (≥ 160/100 mm Hg), heart failure, obvious nutritional deficiencies or using corticosteroids were not included into the study. All participants gave written, informed consent to the training program.

The training program lasted 4 months and consisted of two 45-minute sessions of the Chinese Tai Chi exercises weekly. The training was aimed at increasing and maintaining fitness, especially joint mobility, coordination of movements (quickness and precision of movements), and body balance. The intensity of exercises was chosen so that the heart rate did not exceed 110–120 beats per minute. The heart rate was monitored during each exercise session with a heart rate monitor (Accurex Plus, Polar, Finland). The training program included 5 complementary elements: warm-up exercises (stretching and balance exercises), Qi gong (the order of exercises were constant), meditation standing (using Xinghi technique), relaxation massage in pairs (about 2 minutes) and elements of Tai Chi Chuan (5 sequences of movements chosen from the simplified 24-form). Each session was led by a certified Tai Chi instructor. Subjects were recommended not to change their nutrition habits or habitual physical activity, including avoiding of performing Tai Chi between the supervised sessions, during the study period.

Before entering the training program, and after completing it, anthropometric measurements were taken, body mass index (BMI) was calculated and body composition was assessed with an electric bioimpedance method (101/S analyzer, Akern, Italy).

Blood samples for biochemical analyses were taken from the antecubital vein after an overnight fast (between 7 AM and 8 AM) and centrifuged immediately to separate serum from erythrocytes. Serum, plasma and red blood cell hemolyzate samples were stored at -70°C until analysis.

Total antioxidant status (TAS) of plasma was measured using a commercially available assay (Randox Laboratorie Ltd., UK). Concentrations of thiobarbituric acid reactive substances (TBARS) in plasma were estimated using a spectrophotometric method with chromogen extraction with n-butanol, described by Buege and Aust (17). The reduced glutathione (GSH) content and glutathione peroxidase (GPx) activity in hemolyzed red blood cells were measured with commercially available assays (BIOXYTECH® GSH–400™ and BIOXYTECH® GPx–340™ respectively, Oxis International, Inc., USA). GSH concentration and GPx activity were calculated per 1 gram of hemoglobin. The concentration of hemoglobin in the red blood cell hemolyzate was assessed with HemoCue Blood Hemoglobin analyzer (Sweden).

Serum concentrations of glucose, HDL-cholesterol and triglycerides were assessed with commercially available assays (Cormay, Poland). Insulin levels were evaluated with an immunoradiometric assay kit (INS-IRMA, BioSource S.A., Belgium). Atherogenic index of plasma (AIP) was calculated from HDL-cholesterol and triglycerides levels according to the method proposed by Dobiasova (18). The insulin sensitivity index $HOMA_{IR}$ was calculated using the mathematical formula described by Matthews and collaborators (19). Additionally, we calculated the triglycerides (TG) to HDL-cholesterol (TG-C) ratio (both variables expressed in mg/dl), being another good predictor of insulin sensitivity (20).

The protocol of the study was accepted by the local Ethics Committee.

Statistical analysis

All data are expressed as mean ± standard deviation (SD). All statistical analyses were performed with STATISTICA v. 6.0 software package. The differences between paired variables were investigated with Wilcoxon test. Spearman's rank analysis was used to calculate correlation coefficients. P value < 0.05 was considered significant.

Results

Anthropometric and biochemical parameters before and after the training program are shown in Table 1. No significant changes in the anthropometric parameters (body weight, body mass index and fat mass) were found.

Glucose levels, insulin concentrations, and insulin sensitivity index $HOMA_{IR}$ and ratio of TG/HDL-C were significantly lower after the 4-month Tai Chi training ($p<0.01$; Table 1). Serum lipid profile improved over the study period (triglycerides levels decreased ($p<0.01$) and HDL-cholesterol concentrations increased ($p<0.05$), and atherogenic index of plasma decreased significantly ($p<0.01$). Activity of glutathione peroxidase in red blood cells increased significantly following the training program ($p<0.01$), while the reduced glutathione content in red blood cells and total antioxidant status of plasma remained unchanged. Concentrations of thiobarbituric acid reactive substances in plasma increased significantly over the study period ($p<0.01$).

Spearman's rank analysis showed negative correlation between GSH concentrations and insulin levels ($r = - 0.46$; $p<0.01$), between GSH levels and $HOMA_{IR}$ ($r = - 0.40$; $p<0.05$), and between GSH concentrations and atherogenic index of plasma ($r = - 0.43$; $p<0.05$) and TG/HDL-C ratio ($r = -0.42$; $p<0.05$). A positive correlation between GSH concentrations and HDL-cholesterol levels was found ($r = 0.56$; $p<0.01$)

Table 1. *Anthropometric and biochemical parameters in the elderly men before and after the 4-month Tai Chi training (x ± SD)*

	Before training	After training	Wilcoxon test
Age (years)	68.8 ± 5.90		
Body height (cm)	168.9 ± 6.40		
Body mass (kg)	82.6 ± 13.3	82.6 ± 13.21	1.000
BMI (kg/m^2)	28.8 ± 3.54	27.8 ± 3.65	0.690
FM (kg)	26.9 ± 7.47	26.1 ± 7.50	0.184
Glucose (mmol·L^{-1})	6.22 ± 1.10	5.61 ± 1.13	0.01**
Insulin (μU/m·L^{-1})	11.36 ± 3.70	8.27 ± 2.83	0.01**
TG (mmol·L^{-1})	1.18 ± 0.41	1.07 ± 0.28	0.01**
HDL-C (mmol·L^{-1})	1.36 ± 0.29	1.41 ± 0.27	0.05*
$HOMA_{IR}$	3.15 ± 1.24	2.29 ± 0.78	0.01**
GSH (μmol·gHb^{-1})	2.96 ± 0.66	3.0 ± 1.11	0.792
GPx (U·gHb^{-1})	22.97 ± 4.91	33.32 ± 12.98	0.01**
TAS (mmol·L^{-1})	0.92 ± 0.26	0.88 ± 0.28	0.291
TBARS(μmol·L^{-1})	3.0 ± 0.76	3.66 ± 0.80	0.01**
TG/HDL-C (mg·dL^{-1})	2.25 ± 0.31	1.89 ± 0.29	0.01 **
AIP	–0.08 ± 0.197	-0.12 ± 0.15	0.01**

AIP = atherogenic index of plasma; BMI = body mass index; FM = fat mass; GPx = glutathione peroxidase activity; GSH = reduced glutathione; HDL = high-density lipoproteins; $HOMA_{IR}$ = Homeostasis Model Assessment Insulin Resistance; TAS = total antioxidant status; TBARS = thiobarbituric acid reactive substances; TG = triglycerides;
*P < 0.05; **P < 0.01

Discussion

Changes in the lifestyle, diet modification and systematic physical activity delay the development of metabolic disturbances leading to type 2 diabetes and ischemic heart disease, and are probably the best method of maintaining good health status while aging (21-22). The results of several studies (5,13,23,24) showed a significant relationship between systematic, aerobic training leading to the reduction of fat mass, and improvement in insulin sensitivity as well as glucose and lipid metabolism parameters in the elderly.

During the past 20 years, Tai Chi Chuan (in most of the English literature called Tai Chi) has spread throughout Western countries and is offered in many local community services. It represents a class of exercise that differs from the routine strengthening and stretching programmers currently employed in physical medicine. A variety of health-related benefits for older people are attributed to Tai Chi, including improved balance control, improved cardiorespiratory functions, enhanced psychosocial well-being and reduced risk of falling (16). However, little is known about the influence of Tai Chi on metabolic risk factors for atherosclerosis (25).

In the present study, we have found a significant decrease in glucose and insulin levels in healthy, elderly men participating in 4-month Tai Chi training ($p<0.01$; Table1). The insulin resistance index $HOMA_{IR}$ decreased significantly from 3.15 ± 1.24 to 2.29 ± 0.78; Taking into account that the cutoff point for diagnosis of insulin resistance in adults based on $HOMA_{IR}$ index is > 2.5 proposed by Wallace (26), the overweight participants were insulin-resistant at baseline and insulin sensitive after Tai Chi training. In accordance with findings concerning $HOMA_{IR}$, we observed a significant decrease in TG/HDL-C ratio (2.25 at baseline vs 1.89 after the training ($p<0.01$; Table 1). TG/HDL-C ratio has a strong association with insulin resistance and cardiovascular mortality, and may be regarded as a reasonable surrogate for insulin resistance (27,28). Of note, the improvement in insulin sensitivity could not have resulted from the reduction in the fat mass, because the fat mass and body mass remained unchanged over the study period (Table 1).

Hyperglycemia is one of the most important factors increasing oxidative stress. Reactions such as glucose autooxidation, glycation of proteins and formation of advanced glycation end products (AGEs) are potent sources of reactive oxygen species (29). We have found a significant correlation between glucose metabolism parameters and the level of the most important intracellular antioxidant – reduced glutathione. GSH concentrations in red blood cells hemolyzate correlated negatively with serum insulin levels ($r = - 0.46$; $p<0.01$), and insulin resistance index $HOMA_{IR}$ ($r = - 0.40$; $p<0.05$).

We have found a significant increase in the activity of glutathione peroxidase, an important intracellular antioxidant enzyme, following the 4-month aerobic

training. No significant changes in other antioxidant parameters were found: concentrations of GSH in the red blood cells and total antioxidant status of plasma remained unchanged. Interestingly, plasma levels of thiobarbituric reactive substances, being a marker of oxidative stress, increased significantly over the study period. These seemingly conflicting findings may be explained by the concept of hormesis proposed by Masoro (30). According to this theory, a subtle oxidative stress (associated with systematic physical activity for example) induces adaptative changes in different types of cells and increases their antioxidant potential (31). As a result cells become less susceptible to damages associated with an acute oxidative stress. It is tempting to speculate that a slight, chronic oxidative stress associated with the training (as indicated by the slight increase in TBARS levels), was a stimulus that increased antioxidant potential in the red blood cells (increased activity of GPx). Data concerning influence of regular aerobic exercise on physiological antioxidant defense in healthy elderly men are inconsistent. Several studies found an increase of antioxidants after training (31-33). On the other hand, either no changes or even a decrease in antioxidant potential was shown in other works (34-35). Of note, we have found no studies concerning the influence of Tai Chi on oxidative stress and antioxidant status in the elderly.

It is well known that insulin resistance, an important risk factor for atherosclerosis, is accompanied by atherogenic lipoprotein phenotype, which considerably enhances the risk (36). To assess the balance between lipid risk and protective factors, Luc et al. (20) proposed calculation of triglyceride/HDL-cholesterol ratio. The usefulness of this ratio was confirmed in several cross-sectional studies. Dobiasova (18) introduced atherogenic index of plasma (AIP), which is the logarithm of the TG/HDL-cholesterol ratio. This modification resulted in significant decrease in the variability of the parameter. According to Reaven (37), AIP is the best parameter to assess the risk associated with atherogenic lipid profile. Tan (38) suggests that this parameter may be useful while monitoring the efficacy of the antiatherogenic medication. In our study, we observed favorable changes in lipid metabolism in healthy, elderly men participating in 4-month aerobic Tai-Chi training (Table 1). Serum HDL-cholesterol levels increased, while triglycerides concentrations, TG/HDL-C ratio, and atherogenic index of plasma decreased significantly following the training program ($P < 0.01$). We have also found a negative correlation between GSH content and AIP ($r = - 0.43$; $P < 0.05$), and there was a positive correlation between GSH and HDL-cholesterol ($r = 0.56$; $P < 0.01$). Increase in concentrations of high-density lipoproteins is maybe associated with an increase in the activity of paraoxonase, which is an important antioxidant enzyme that prevents oxidative modification of the light-density lipoproteins (LDL).

Conclusions

4-month physical activity based on Tai Chi Chuan exercises favorably influences glucose and lipid metabolism and decreases insulin resistance in overweight, elderly men even despite the lack of body mass and fat mass reduction. It may also enhance antioxidant potential. The improvement in glucose and lipid metabolism, as well as the antioxidant defense system, may significantly decrease the risk of atherosclerosis in elderly men.

References

1. Xu H, Barnes GT, Yang Q, et al. Chronic inflammation in fat pays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest* 2003; 112: 1821-30.
2. Barbieri M, Bonafe M, Franceschi C, et al. Insulin/IGF-1-signaling pathway: an evolutionarily conserved mechanism of longevity from yeast to humans. *Am J Physiol Endocrinol Metab* 2003; 285: E1064-71.
3. Gumbiner B, Thorburn AW, Ditzler TM, et al. Role of impaired intracellular glucose metabolism in the insulin resistance of ageing. *Metabolism* 1992; 41: 1115-21.
4. Furukawa S, Fujita T, Shimabukuro M, et at. Increased oxidative stress in obesity and its impact on metabolic syndrome. *J Clin Invest* 2004; 114: 1752-61.
5. Barbieri M, Rizzo MR, Manzella D, et al. Glucose regulation and oxidative stress in healthy centenarians. *Exp Gerontol* 2003; 38: 137-43.
6. Ferrannini E, Vichi S, Beck-Nielsen H, et al. European Group for the Study of Insulin Resistance (EGIR) Insulin action and age. *Diabetes* 1996; 45: 947-53.
7. Gil L, Siems W, Mazurek B, et al. Age-associated analysis of oxidative stress parameters in human plasma and erythrocytes. *Free Radic Res* 2006; 40(5): 495-505.
8. Stadtman ER. Importance of individuality in oxidative stress and aging. *Free Radic Biol Med* 2002; 33: 597-604.
9. Matsuoka T, Kajimato Y, Watada H, et al. Glycation-depentent , reactive oxygen species – mediated suppression of the insulin gene promoter activity in HIT cells. *J Clin Invest* 1997; 99: 144-50.
10. Paolisso G, Tagliamonte MR, Rizzo MR, et al. Advancing age and insulin resistance: new facts about an ancient history. *Eur J Clin Invest* 1999; 29: 758-69.
11. Berr C. Cognitive impairment and oxidative stress in the elderly: Results of epidemiological studies. *BioFactors* 2000; 13: 205-9.
12. Tokuyama K, Higaki Y, Fujitani J, et al. Intravenous glucose tolerance test-derived glucose effectiveness in physically trained humans. *Am J Physiol* 1993; 265: E298-303.
13. Pilaczyńska-Szcześniak Ł, Maciaszek J, Deskur-Śmielecka E, et al. The effect of Tai-Chi training on surrogate index of insulin resistance (Homa $_{IR}$) in elderly subjects. *Human Movement* 2005; 6(2): 98-103.
14. Dylewicz P, Przywarska I, Szcześniak Ł The influence of short-term endurance training on the blood level, binding and degradation of ^{125}I-insulin by erythrocyte receptors in patients after myocardial infraction. *J Cardiopul Rehab* 1999; 9: 98-105.
15. Miyazaki H, Oh-ishi S, Ookawara T, et al. Strenuous endurance training in humans reduces oxidative stress following exhausting exercise. *Eur J Appl Physiol* 2001; 84: 1-6.
16. Li F, Harmer P, McAuley E, et al. An evaluation of the effects of Tai-Chi exercise on physical function among older persons: a randomized controlled trial. *Am Behav Med* 2001; 23: 139-46.

17. Buege J, Aust SD. The thiobarbituric acid assy. In: Rice-Evans CA, Diplock AT, Symons MCR, eds. Techniques in Free Radical Research. Amsterdam: Elservier, 1991: 147-8.
18. Dobiasowa M, Frohlich J. The plasma parameter log (TG/HDL-C) as atherogenic index: correlation with lipoprotein particle size and esterification rate in apoB-lipoprotein-depleted plasma (FER_{HDL}) *Clin Biochem* 2001; 34: 583-8.
19. Matthews DR, Hosker JP, Rudenski AS, et at. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentration in man. *Diabetologia* 1985; 28: 412-9.
20. Luc G, Bard JM, Ferrieres J, et al. Value of HDL cholesterol, apolipoprotein A-I, lipoprotein A-II, and lipoprotein A-I/AII in prediction of coronary heart disease: the PRIME study. Prospective Epidemiological Study of Myocardial Infarction. *Arterioscler Thromb Vasc Biol* 2002; 22: 1155-61.
21. Ryan AS. Insulin resistance with aging. Effects of diet and exercise. *Sports Med* 2000; 30: 327-46.
22. Short KR, Vinton JL, Bigelow ML, et al. Impact of aerobic exercise training on age-related changes in insulin sensitivity and muscle oxidative capacity. *Diabetes* 2003; 52: 1888-96.
23. Ilhan N, Kamanli A, Ozmerdiveneli R., et at. Variable effects of exercise intensity on reduced glutathione, thiobarbituric acid reactive substance, and glucose concentration. Arch Med Res 2004; 35: 294-300.
24. Fiatarone Singh MA. Exercise and aging. *Clin Geriatr Med* 2004; 20: 201-21.
25. Yeh S-H, Chuang H, Lin L-W. Tai Chi Chuan exercise decreases A1C leves along with increase of regulatory T-cells and decrease of cytotoxic T-cell population in type 2 diabetic patients. *Diabetes Care* 2007; 30: 716-8.
26. Wallace TM, Matthews DR. The assessment of insulin resistance in man. *Diabetic Med* 2002; 19: 527-34.
27. McLaughlin T, Abbasi F, Cheal K, et al. Use of metabolic markers to identify overweight individuals who are insulin resistant. *Ann Intern Med* 2003;139: 802-9.
28. McLaughlin T, Reaven G, Abbasi F, et al. Is there a simple way to identify insulin - resistant individuals at increased risk of cardiovascular disease? *Am J Cardiol* 2005; 96(3): 399-404.
29. Ceriello A. Oxidative stress and glycemic regulation. *Metabolism* 2000; 49: 27-9.
30. Masoro EJ. Hormesis and the antiaging action of dietary restriction. *Exp Gerontol* 1998; 33: 61-6.
31. Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. *Nature* 2000; 408: 239-47.
32. Lawler JM, Powers SK. Oxidative stress, antioxidant status, and the contracting diafragm. *Can J Appl Physiol* 1998; 23:23-55.
33. Nakatani K, Komatsu M, Kato T, et al. Habitual exercise induced resistance to oxidative stress. *FreeRad Res* 2005; 39 (9): 905-11.
34. Meijer EP, Coolen SAJ, Westerterp KP. Exercise training and oxidative stress in the elderly as measured by antipyrine hydroxylation products. *Free Rad Res* 2001; 35:435-43.
35. Elosua R, MolinaL, Fito M, et al. Response of oxidative stress biomarkers to a 16-week aerobic physical activity program, and to acute physical activity, in healthy young men and women. *Atherosclerosis* 2003; 167: 327-34.
36. Krauss RM. Lipids and lipoproteins in patients with type 2 diabetes. *Diabetes Care* 2004; 27: 1496-504.
37. Reaven GM, Chen YD, Jeppesen J, et al. Insulin resistance and hyperinsulinemia in individuals with small, dense low density lipoprotein particles. *J Clin Invest* 1993; 92:141-6.
38. Tan MH, Johns D, Glazer NB. Pioglitazone reduces atherogenic index of plasma in patients with type 2 diabetes. *Clin Chem* 2004; 50: 1184-8.

Received: March 29, 2007
Accepted : June 29, 2007
Published: June 30, 2007

Address for correspondence
Joanna Karolkiewicz, PhD
Chair of Hygiene
University School of Physical Education
Ul. Królowej Jadwigi 27/39
61-871 Poznań
Poland
tel. +4861 8355177
e-mail: karolkiewicz@awf.poznan.pl

Author's contribution
A – Study Design
B – Data Collection
C – Statistical Analysis
D – Data Interpretation
E – Manuscript Preparation
F – Literature Search
G – Funds Collection