DOPING IN SPORT: NEW DEVELOPMENTS

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ABSTRACT

Gene doping is defined by the World Anti-Doping Agency (WADA) as "the non-therapeutic use of genes, genetic elements and/or cells that have the capacity to enhance athletic performance." The rapid development of molecular biology has enabled not only treatment of many diseases, but also improvement of athletes' fitness. Gene therapy methods can be used to modify the athlete's body by inserting genes into the target tissue. It is very possible that in near future, many genes will be used in gene doping, e.g. erythropoietin, growth hormone, insulin-like growth hormone and vascular endothelial growth factor. Functional tests conducted by many independent laboratories proved that products of these genes exert a crucial influence on the body's adaptation to exercise. The risk of gene doping is enormous. Gene therapy is currently in the phase of clinical tests so it is impossible to predict what kind of side effects it may produce. Studies on animal models showed that the uncontrolled transgene expression and insertional mutagenesis can even lead to death. At present the detection of gene doping is very difficult for a variety of reasons. The main problem is the identification of the transgene and endogenously produced protein. The only possible detection is the biopsy of the target tissue, where the exogenous genes were inserted.

Key words: gene doping, performance enhancement, gene transfer, doping detection

Introduction

One of the most serious problems of present-day competitive sport is the increasing abuse of various performance-enhancing substances. Doping is not a product of modern times; illegal stimulants were already used in antiquity. The first recorded case of using substances enhancing athletes' physical performance dates back to the Olympic Games in the 3rd century BC. The use of doping was also known in ancient Rome and Egypt. The first banned substances were of natural origin and included, for instance, a beverage from donkey hooves, or dried figs [1]. In the 19th century long-distance swimmers and cyclists were reported to use such performance enhancing substances as caffeine, strychnine, ether, alcohol or oxygen. The state of knowledge of the human physiology and effects of different substances on metabolism of human cells was fairly limited at that time and athletes' abuse of the mentioned compounds led sometimes to death. The first recorded lethal case was cyclist Arthur Linton who died in 1896 after having taken strychnine. The absence of drug tests made many athletes enhance their performance with impunity. Taking stimulants, however, not always yielded favorable results and often led to serious health problems.

In the case of present-day athletes one may gain an impression that using "ordinary" stimulants is not sufficient. The development of medicine and molecular biology makes it possible to use doping adjusted to individual physiological parameters important for a given sport. The number of athletes ready to violate the rules of fair play to achieve better results increases constantly. Many of them are ready to risk their health, or even life, to take first place. Attaining a desired aim takes priority over health consequences of using doping substances.

At present, the list of banned substances taken orally, intravenously or intramuscularly includes a few dozen items. In recent years some recombinant substances[1] that have been successfully used in treatment of many

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[1] Recombinant substances are natural compounds present in the human body, which are manufactured synthetically, e.g. growth hormone, erythropoietin and insulin.

diseases have been often administered as illegal stimulants. The first recombinant substance was insulin, produced in bacteria host cells following the insertion of a viral vector carrying an insulin-encoding gene. It became registered in the United States in 1982 as Humulin. Recombinant substances can enhance the muscle function, aerobic capacity or the body's regeneration. They are manufactured using prokaryotic or eukaryotic expression systems. Their production consists of constructing expression vectors, which are fragments of genomes (e.g. bacterial, Saccharomyces or mammalian cell lines), to introduce and express a specific gene into a target cell. Once the expression vector is inside the cell, a protein encoded by the gene is produced by the cellular transcription and translation machinery [2]. The recombinant substances are still very difficult to detect, and an increase in their abuse by athletes can be expected in the future. The commercialization of new pharmaceuticals manufactured with the aid of biotechnology will lead to their wider accessibility.

A form of doping which has recently become very attractive to some athletes is gene doping based on gene therapy methods, which allows direct insertion of genes into the athlete's body. The products of such genes, e.g. proteins, can be used as a substance enhancing athletic performance. The long-term production of such protein in the target tissue increases its concentration and activity. Gene doping methods include:
- insertion of a specific gene into the target tissue;
- administration of substances enhancing gene expression.

The prospects of gene doping can be very attractive to athletes, as for the time being its detection remains virtually impossible.

Gene doping is becoming a serious hazard. In 2005 the World Anti-Doping Agency (WADA) defined gene doping as "the non-therapeutic use of genes, genetic elements and/or cells or modification of gene expression that have the capacity to enhance athletic performance." The list of prohibited substances tested for gene doping becomes longer each year; the most popular substances include growth hormone (GH), erythropoietin (EPO), insulin, insulin-like growth factor (IFG-I), hemoglobin-based oxygen carriers (HBCOs), alpha-actinin 3 (ACTN3), angiotensin convertase (ACE), hypoxia inducible factor (HIF-1α), delta peroxisome proliferative activated receptor delta (PPAR), and endothelial growth factor (VEGF).

Gene insertion techniques used in gene therapy can be divided into:

- biological: through viral vectors;
- physical: through injection or gene gun;
- chemical: through liposomes.

The most effective are methods using viral vectors, which can modify both prokaryotic cells (bacteria) and eukaryotic cells (yeasts, insect cells, mammalian cell lines). They usually involve deletion of a part of the viral genome critical for viral replication. Such a recombinant virus can efficiently infect cells which are capable of transgene expression. The most frequently applied viral vectors are retroviruses, adenoviruses, Herpes simplex viruses and adeno-associated viruses (AAV).

An ideal viral vector should:
- be able to penetrate both prokaryotic and eukaryotic cells;
- easily integrate into the host genome;
- not cause any responses of the immune system;
- feature a high level of expression of the carried gene;
- synthesize proteins of low toxicity;
- be capable of regulating gene expression.

The main advantages of viral vectors include long-lasting expression of the transduced gene and cellular tropism. However, such a significant intervention in the human body may lead to severe complications like:
- insertional mutagenesis, associated with the applied viral vector system, type of transformed cell, location of vector integration, and transgene expression;
- development of an infectious virus;
- excessively long or uncontrolled transgene expression.

During insertion of a gene into the organism, its expression should be controlled to prevent excessive amounts of protein from accumulating. Otherwise an uncontrolled cell division, cell toxication or poisoning of the entire body may occur.

Gene therapy involves many risks related to the ways of gene insertion into the target tissue, immunological response to the viral vector, or insertional mutations. The history of gene therapy knows cases of patients' deaths or development of leukemia, e.g. death of a patient suffering from the deficiency of transcarbamoilase due to transduction of an unmutated gene; or induction of insertional mutation by a retroviral vector resulting in leukemia in 2 out of 11 patients suffering from X chromosome-related acute complex immunological deficiency [3].

The effects of application of such methods by athletes willing to "perfect" their bodies are very difficult

to predict. There are alarming reports about studies into gene transduction into human tissues and gene expression resulting in long-lasting protein overexpression. Observing the increasing tendency among athletes to constantly enhance their performance by all means, one can expect that they would reach for gene doping, unaware of its hazards. Intensive research into gene doping detection has so far brought no successful results. Gene doping detection is exceptionally difficult as the inserted gene causes an increase in the concentration of a specific substance only in the target tissue. Thus any blood or urine tests are not able to detect it. It is also highly probable that a transgene product will be identical to a naturally synthesized protein, and the detection of differences between such two products will require a great quantity of detailed tests. Another question is whether a higher concentration of a given factor is a result of training, doping or athlete's genetic predispositions which can lead to its increased expression.

Selected non-viral techniques of gene insertion

Electroporation

Electroporation consists of application of a high voltage current to target cells. It opens the pores in the cell membrane through which exogenous DNA can enter the cell.

Gene gun

A gene gun is a device for injecting cells with genetic information. It uses particles of a heavy metal (gold, silver, tungsten) coated with plasmid DNA. The DNA coated particle is delivered to the target tissue with the aid of a helium propellant and the DNA is released into the cytoplasm. The inserted DNA then migrates into the cell nucleus where it becomes integrated into the genome DNA.

Liposomes

Liposomes are spherical vesicles composed of a bilayer membrane, whose structure resembles the membrane of animal cells. The delivery of DNA into a cell consists of a fusion between the liposome and the cell membrane. The amount of DNA delivered this way is far smaller than by using viral vectors; however, the liposomes have been considered by researchers for drug delivery in treatment of cancer and a number of genetic disorders.

Prohibited substances used in sport doping

Growth hormone (GH)

One of the most commonly abused substances in sport over the last decades has been undoubtedly growth hormone, also known as somatotrophine. GH is a protein hormone, which is secreted in large pulses from the anterior pituitary. The GH secretion is stimulated during sleep, fever, physical exercise, stress as well as by some amino acids (leucine, arginine) and hormones (estrogens and androgens). The GH level also increases in hyperthyroidism. Long and intense physical exercise can elevate GH secretion up to ten times [4]. The inhibitors of GH secretion include obesity and hyperglycemia.

The peak GH secretion takes place in puberty and then it decreases with age. GH affects a number of different tissues; however, it exerts the greatest influence on the bone tissue, cartilage, skeletal muscle, adipose tissue, liver, kidneys and the immune system. Somatotrophin has anabolic activity. Its protein synthesis potential is comparable to that of testosterone. GH promotes lipolysis, enhances calcium retention and bone growth and mineralization by stimulating chondrocytes to synthesize insulin-like growth factor (IGF-I), which in turn enhances the synthesis of collagen and mucopolysaccharides. It also promotes the synthesis of IGF-I in the liver. GH is regarded as the agonist of insulin. Some factors, e.g. stress or physical exercise, activate the central nervous system which sends impulses to the hypothalamus. Depending on the type of impulse the hypothalamus cells start synthesizing the growth hormone releasing hormone (GHRH) or growth hormone inhibiting hormone, i.e. somatostatine (SS). These factors reach the anterior pituitary and stimulate or inhibit GH secretion. GH receptors are located in the majority of human tissues. The contact between GH and a receptor results in dimerization of the former and stimulation of the intracellular domain of the receptor tyrosine kinase. The signal transduction consists of protein phosphorylation and, in consequence, of regulation of transcription of specific genes. GH secretion can be also stimulated by ghrelin – a short peptide produced in the hypothalamus, anterior pituitary and stomach.

About 50% of GH in the blood is bound to the growth hormone binding protein (GHBP). The GHBP

level in serum depends on a number of factors, e.g. age, nutritional status, and the levels of growth hormone, insulin and sex hormones.

Important mediators in GH activity are insulin-like growth factors (IGF-I, IGF-II) synthesized in the liver stimulated by GH. The activity of IGFs can be of autocrine and paracrine character, e.g. in fibroblasts, myoblasts, chondroblats, osteoblats and brain or renal cells.

In the late 1950s GH from the pituitary of deceased persons was first used for treatment of somatorophin deficiency in children. In 1989 recombinant human growth hormone (rhGH) was developed. The rhGH treatment of patients with GH deficiency increases the muscle mass and lean body mass, reduces the adipose tissue and enhances the functioning of the heart and kidneys [4].

Growth hormone became commercially available as an ergogenic aid in 1988 and soon became immensely popular among athletes who were interested in the enhancement of their training quality. The attractiveness of GH relied on a conviction that the hormone strengthened tendons, accelerated tissue regeneration, increased muscle mass and strength and reduced the fat deposition. Its main advantages were anabolic activity, accessibility, small risk of side effects and impossibility to detect [5].

Growth hormone is mainly abused by strength sports athletes, sprinters as well as endurance sports athletes and soccer players. GH is often used by women as it entails no risk of androgenic side effects [4]. A number of outstanding athletes were known to use GH doping, e.g. Ben Johnson, who was stripped of his Olympic gold medal from Seoul in 1988 after having admitted taking GH in combination with anaerobic steroids for many years [5].

Despite the conviction about the absolute safety of GH use, there are indications that a long-term administration of human growth hormone can increase the risk of diabetes, retention of fluids, joint and muscle pain, hypertension, cardiomyopathy, osteoporosis, irregular menstruation, impotence and elevated HDL cholesterol [6].

A few independent laboratory tests failed to confirm the observation of GH greatly enhancing muscle strength and mass. Taking large doses of GH does not enhance exercise-induced protein synthesis. It leads to an increase of the lean body mass in result of adipose tissue reduction, increasing total body water and the level of total protein synthesis in the body. These data

concern, however, the entire body rather than individual body parts. An increase in muscle strength was not observed due to GH administration [4].

Insulin-like growth factor I (IGF-I)

The insulin-like growth factor I (IGF-I) is a mediator of growth hormone which affects almost all tissues in the human body. Six IGF binding proteins have been identified in serum as well as an acid-labile subunit (ALS). IGF-I affects the cells through the receptor tyrosine kinase but it can also bind with an insulin receptor and cause hypoglycemic effects. IGF-I plays a key role in GH regulatory processes. The local synthesis of IGF-I leading to its autocrine and paracrine secretion is an indication that IGF-I is a factor inducing a variety of metabolic processes [5]. The blood concentration of IGF-I is related to age, sex, diet (low-calorie and low-protein diet decrease the IGF-I level) as well as the levels of insulin, parathyroid hormones, sex hormones (estrogens decrease and androgens increase the IGF-I level). In diabetes and pregnancy the IGF-I synthesis decreases, but it rises in regenerating tissues (especially in skeletal muscle). A higher IGF-I synthesis was observed in the course of some malignant diseases, e.g. prostate, breast and colon cancer. The spectrum of IGF-I activity includes:

- stimulation of cell growth and development in bone, cartilage, muscle, hematopoetic system, kidneys and pancreas;
- stimulation of growth;
- activation of protein synthesis and glucose and glycogen uptake;
- inhibition of degradation of muscle proteins;
- regeneration of tissues;
- antiapoptic effects (in reaction to hypoxia);
- stimulation of acetylocholine secretion;
- regulation of the cell growth cycle;
- modulation of immunological response (affecting the synthesis of cytokines, and immune system cells) [6].

The clinical use of recombinant human IGF-I (rhIGF-I) is based on its regulatory properties. The rhIGF-I regulates glucose homeostasis through regulation of insulin activity and it has been used in treatment of diabetes mellitus type 1 and insulin resistance for about twenty years. The administration of IGF-I in patients with diabetes mellitus type 2 and acute insensitivity to insulin has also yielded positive test results. The

influence of endogenous IGF-I on glucose metabolism is not clear: it either directly lowers the glucose activity, or directly increases insulin sensitivity [7].

The control of IGF-I expression is multilevel. The IGF-I expression is controlled by hormones, mainly glycocorticoids, which cause a decrease of IGF-I expression in osteoblasts and of type I collagen expression as well as an increase of type III collagen expression. They also induce apoptosis in many types of cells. Research has shown that cortisol lowers IGF-I expression for 50%, whereas parathormone and prostaglandin E2 (PGE2) stimulate it [8, 9].

Endogenous IGF-I has three protein isoforms:
• IGF I-Ea synthesized in skeletal muscle, very similar to the main IGF-I form produced in the liver;
• Mechano-Growth Factor (MGF) produced in skeletal muscle;
• IGF I-Eb produced in the liver; its role in muscle remains unknown.

Exercise-induced MGF expression most likely leads to the activation of satellite cells responsible for the formation of new muscle fibers. MGF plays a significant role in muscle hypertrophy following training or muscle damage [10]. In young people the MGF expression occurs after the first strength training session. In older people it comes much later: only after five weeks training did the amount of mRNA MGF increase for 163%. Strength training-induced MGF expression increases even for 456% following the administration of rhGH in elderly men [11]. It proves that only the combination of physical exercise and administration of rhGH can result in muscle hypertrophy. Most studies have indicated that rhGH administered in non-training subjects fails to enhance muscle strength [10]. One of results of strength training was also the expression of IGF-Eb; however, the precise role of this protein in muscle remains unknown.

IGF-I can be abused as a stimulant due to some of its properties:
• hypertrophic adaptation of muscles to exercise, i.e. working hypertrophy;
• potential contribution to adaptation of the cardiac muscle to exercise;
• participation in glucose metabolism;
• affecting the ion balance.

IGF-I can be regarded as an attractive doping substance due to its role in the Growth Hormone/IGF-I axis in glucose metabolism. Clinically administered GH and IGF-I induce changes in glucose homeostasis and resistance to insulin. The role of IGF-I is similar to that of insulin, whereas GH is antagonistic. The precise mechanism of these processes remains largely unknown; however, an increased GH secretion lowers the insulin blood level, which leads to IGF-I synthesis in the liver [12]. Studies on mice showed that the lack of IGF-I gene resulted in a decrease of IGF-I in circulation, higher blood levels of GH and insulin and reduced or absence of insulin sensitivity. Insulin insensitivity was shown to be muscle-specific and associated with the lack of activation of insulin receptor and insulin-receptor substrate. The insertion of recombinant human IGF-I decreased the level of insulin in blood and increased insulin sensitivity [12]. These studies confirm that IGF-I is an important factor regulating the glucose concentration in blood, which significantly affects the body's adaptation to physical exercise.

Each kind of strength, endurance or speed training significantly loads the myocardium. There are currently a number of studies being conducted on rats which are to reveal whether IGF-I can influence the adaptation of the cardiac muscle to physical exercise. The insertion of exogenous IGF-I increases the amount of mRNA of the insulin-like growth factor I receptor after six weeks of swimming training. The training caused an elevated myocardial IGF-I mRNA expression in rats with and without transduced exogenous IGF-I [13]. These results point to the fact that the role of IGF-I in the body's adaptation to intensive exercise is not merely confined to the muscle tissue but is related with all IGF-I sensitive tissues.

An intramuscular, intravenous injection or oral administration of IGF-I or other doping substances is easily detectable in blood or urine tests. Thus, gene therapy researchers pointed to the fact that gene transfer could be also used for enhancement of athletic strength and performance. Numerous tests were carried out aimed at the IGF-I gene transfer via a viral vector into the muscle tissue of mice with Duchenne muscular dystrophy [14]. The inserted IGF-I gene was only expressed in skeletal muscle cells, but there was no increase in the concentration of IGF-I in blood or urine. The results were remarkable: an increase in muscle mass of 15–30% was observed in the mice infected with the recombinant virus. Other tests show that in mice with permanent IGF-I gene overexpression the skeletal muscle mass was increased for about 20–50% and their regenerative capabilities were similar to those of younger organisms. The muscle regeneration consisted of reconstruction of the

cell membrane of muscle fibers and formation of new myofibrils in an existing muscle fibre. In mice with heavy dystrophy the regenerative processes were greatly accelerated. The question appeared whether apart from the increase in muscle mass an increase in muscle strength can be also achieved. H. Sweeney's and R. Farrar's teams revealed that an insertion of a vector carrying the IGF-I gene and a few weeks strength training caused a twofold increase in muscle strength. Characteristically, after discontinuation of training the muscle with the transgene returned to its original state much slower than the one without it. Additionally, an increase in muscle strength of 15% was observed in rats unable to move after the IGF-I transfer [14]. These results are very promising as they point to the great significance of IGF-I in muscular hypertrophy and regeneration. For the time being, the conducted tests do not allow clinical application of gene therapy using IGF-I. An insertion of the IGF-I gene into a human body followed by its uncontrolled overexpression may lead to:

- excessive growth of muscle mass inadequate to the strength of bones, tendons and ligaments;
- hampering of blood circulation related to muscular hypetrophy;
- cardiac and thyroid diseases.

Insulin

GH, IGF-I and insulin are the most significant factors in anabolic processes. These proteins act in a synergistic fashion. GH and IGF-I directly stimulate the synthesis of proteins by activating amino acids transporters in the cell membrane. Insulin, on the other hand, inhibits the degradation of proteins and acts synergistically in relation to steroids [5, 15]. The effectiveness of GH activity depends on insulin activity. Administration of GH to patients with a lowered insulin level (suffering from diabetes mellitus type 1 or undergoing hunger cure) was discovered to stimulate catabolic reactions which may lead to diabetic ketoacedosis [5]. Insulin is a substance with a significant impact on the anabolic processes; it is often regarded by many athletes as a drug enhancing training effects through:

- facilitation of glucose uptake which can make the amount of glucose delivered to the cell exceed the cell's physiological demand for glucose, and make the synthesis and storage of large amounts of glycogen possible during physical deconditioning, which would then enhance muscle work during competition;

- transportation of amino acids into muscle cells;
- enhancement of general endurance;
- acceleration of regeneration processes;
- enhancement of the anabolic activity of growth hormone;
- increasing muscle mass and performance through glycogen storage and inhibition of degradation of muscular proteins [5].

Insulin is therefore a highly effective performance-enhancing drug and it can significantly influence an athlete's sport results. Insulin was included in the Prohibited List by the International Olympic Committee. Diabetics are, however, exempted.

Statistical research shows [15] that about 25% of athletes using anabolic androgenic steroids take them in combination with insulin to improve their effects. To avoid hypoglycemia following an intake of insulin athletes consume high-carbonate products. Very few athletes using insulin are aware of the hazards related to its abuse. Taking high doses of insulin may cause hypoglycemia, which if untreated, can lead to a coma or even death.

Erythropoietin (EPO)

Erythropoietin (EPO) is a glycoprotein hormone produced in the renal cortex which significantly stimulates the process of erythropoiesis. It has been one of the most commonly abused drugs by athletes for a few decades. In 1989 the IOC introduced a new category of prohibited substances – protein hormones, which includes recombinant human erythropoietin (rhEPO).

The main function of EPO is regulation of red cells production. Human erythropoietin was the first clinically applied recombinant hematopoetic growth factor [16]. rhEPO is most often manufactured with genetic engineering and biotechnological methods using animal cell lines with a transduced human EPO gene [17]. It is produced in four forms: alpha, beta, omega and delta [16].

The activity of rhEPO rises the quantity of red cells, enhances the uptake of iron, calcium and glucose ions by maturing erythrocytes and elevates the hematocrit level.

The use of rhEPO can significantly affect:
- aerobic capacity;
- VO_{2max};
- ventilatory threshold (VT).

The level of endurance following the insertion of EPO can increase for about 20% [18]. The use of EPO

as a performance-enhancing drug is particularly significant in endurance sports (cycling, swimming, cross-country skiing). After a subcutaneous injection, 20–30% of rhEPO can be detected four days later, and following an intravenous injection 2–3 days later. The EPO level is most likely unrelated to age, sex or the phase of the menstrual cycle, thus setting physiological standards for EPO is not difficult [19]. The erythropoietin abuse can cause a number of serious disorders:

- functional iron deficiency (FID) resulting in a release of hypochromic red cells (HRC) [19];
- increasing risk of thrombosis and pure red cells aplasia (PRCA) [20].

The administration of recombinant erythropoietin from animal cell lines is just one of possible applications of molecular biology methods to enhance the body's aerobic capacity. A number of tests are currently carried out on a possible insertion of homological cDNA via a viral vector into skeletal muscles. Functional tests on macaques revealed that such a transgene featured different patterns of expression than the endogenous form. It resulted in a change of EPO isoelectric mobility requiring higher pH. So far, only animal tests have been conducted, but it can be assumed that the gene expression in a genetically modified human tissue will be different from the endogenous pattern. Since the skeletal muscles constitute a perfect target tissue for the gene transduction, and the effectiveness of its overexpression is very high, the use of rhEPO as a form of gene doping seems vary plausible. The impact of the EPO transgene on the human body requires further research. It may seem that the formation of transgenic muscles should be relatively simple in the age of such remarkable developments in molecular biology, however it still requires a great number of tests on the influence of overexpression of a given protein on the human body. Functional tests carried out in 1997 and 1998, consisting of the transduction of the EPO gene into macaques led to heavy thickening of blood, which can be a cause of myocardial infarction. The EPO transduction can have many possible side effects. On the other hand, the detection of virally inserted EPO may not be that difficult due to the availability of the target tissue and considerable differences between the endogenous and transduced EPO forms [17].

Most recent studies concerning methods of increasing of the EPO level in the body show that stimulation of EPO synthesis may result not only from the insertion of exogenous EPO but also from the activity of hypoxia-inducible factors (HIFs). Hypoxia inhibits the activity of enzymes degenerating HIF-1α and HIF-1β – which permeat to the cell nucleus and stimulate the transcription of EPO gene – as well as of glycolitic enzymes taking part in cellular responses to a lower oxygen concentration. The HIFs can be of potential use in clinical treatment of cancer, inflammations or myocardial infarctions. They can also be used as performance-enhancing drugs increasing the EPO level in blood and thus improving tissue oxygenation. The detection of recombinant HIFs would be very difficult, especially in the case of transduction via viral vectors. The results of insertion can be very severe as the HIFs activate the expression of angiogenic genes and genes modulating cell growth, division and survival. The changes in HIFs expression may also cause tumors.

Potential substances enhancing athletic performance

Apart from the substances discussed above there are many other chemical compounds, whose properties can lead to their abuse as performance-enhancing substances in the near future (Tab. 1)

Table 1. Factors affecting the body's adaptation to exercise

Factor	Function
ACTN3	Structural protein of muscle fibres taking part in regulation of muscular contractions
ACE	Vasoconstriction
HIF-1α	Switching between aerobic and anaerobic metabolism
PPAR delta	Transforming of fast twitch fibres into slow twitch fibres

Alpha-actinin 3 (ACTN3)

Alpha-actinin is a protein belonging to a diverse group of cytoskeletal proteins which also includes dystrophin. Two genes enconde alpha-actinin in humans and are expressed in skeletal muscles: ACTN2 (expressed in all skeletal muscle fibres) and ACTN3 (expressed in fast twitch fibres only). Alpha-actinin 3 is the main structural element of the sarcomere Z-line. It stimulates the formation of fast twitch fibres by cooperating with the signaling pathway – calcineurin. It also stimulates glucose metabolism in fast twitch fibres by binding with an gluconeogenic enzyme (fructose-1,6-

diphosphate). The genetic structure of ACTN3 [21] displays a considerable polymorphism of the stop codon in:

- sprinters,
- endurance sports athletes,
- control group.

The mutation in ACTN3 is a nonsense mutation (CGA – TGA): the arginine codon translates into the stop codon (R577X), which results in the absence of ACTN3 synthesis in fast twitch fibres, which occurs often in endurance athletes. In sprinters the sequence of ACTN3 lacks R577X. It is estimated that this variant is present in both gene copies in about 18% of the world population. These results suggests that the absence of ACT3 is a predisposition to slow but effective muscle contractions, whereas its presence increases the speed of muscle fibre contractions [21]. ACTN3 then is a factor affecting muscle adaptation to exercise, and its polymorphism determines the morphological and functional differences in muscles.

Angiotensin convertase (ACE)

Angiotensin convertase activates angiotensin responsible for vasoconstriction. The ACE gene has two isoforms: D and I. Isoform I was shown to be present in both gene copies in mountaineers who were able to reach the elevation of 7.000 m above sea level with no difficulties, more often than in the general population. A study of rowers also revealed a high incidence of isoform I. These results show that the presence of isoform I of the ACE gene enhances the body's adaptation to strength and endurance training [22].

Hypoxia inducible factor HIF-1α

This transcription factor is a sort of switch between aerobic and anaerobic metabolism. HIF-1α induces modifications in cells so they can adapt to anaerobic conditions. It stimulates glycolysis, angiogenesis and erythropoiesis. The body's endurance depends to a great extent on its adaptation to physical effort in hypoxia conditions. HIF-1α is capable of inducing such adaptation. Knockout studies in mice with removed HIF-1α genes from their skeletal muscle cells revealed exercise-induced tissue-specific changes: decrease or absence of HIF-α, changes in the activity of glycolytic enzymes, low level of lactic acid and muscle damage [23, 24].

Peroxisome proliferative activated receptor delta (PPAR delta)

PPAR delta is a nuclear hormone receptor that bind peroxisome proliferators and control the size and number of peroxisomes produced by cells. It acts as a transcription factor affecting gene expression in muscle fibres. It induces transformation of fast twitch muscle fibres into slow twitch muscle fibres, and is responsible for enhancement of fat oxidation. A transduction of the PPAR delta gene into the skeletal muscle of mice induced:

- an elevated PPAR delta level in slow twitch fibres;
- an increased level of myoglobin in slow twitch fibres;
- an increased concentration of contractile proteins specific to slow twitch fibres;
- a reduced concentration of contractile proteins specific to fast twitch fibres;
- reduced number of fat cells (intensification of oxidative processes, increased tolerance to glucose).

In result, the running endurance of transgenic mice was increased: the running time was longer for 67% and the running distance for 92% [25]. It can be stated that PPAR delta is a significant enhancer of aerobic metabolism of skeletal muscle, which in turn improves the efficiency of muscle work and reduction of the adipose tissue and fatigue.

Vascular endothelial growth factor (VEGF)

VEGF induces:

- proliferation and growth of endothelial cells;
- cell migration;
- an increase in permeability of blood vessels;
- inhibition of apoptosis.

The activity of VEGF leads to a proliferation of blood vessels in organs, which increases the distribution of oxygen to tissues. Clinical tests showed that a transduction of the VEGF gene in patients with atherosclerosis and following myocardial infarction led to formation of new blood vessels. These are preliminary results and the problem of control of VEGF-stimulated proliferation of blood vessels and associated risk of development of neoplasms still remains. An increase in the concentration of VEGF in blood may enhance the performance of the cardiac muscle, improve training effectiveness and delay fatigue. Although the VEGF effects seem very interesting to athletes, research showed an elevated

VEGF level in patients with prostate cancer. The management of risk related to the transduction of the VEGF gene still requires much time and research [26].

Studies on the effects of the above factors on training effectiveness seem to confirm the hypothesis about genetic predispositions to practice a specific sport. Certainly, the environmental factors (appropriate diet and training) determine the athlete's preparation. The identification of genes affecting the crucial parameters in the training process may yield positive results and led to development of training programs specific for individual athletes. On the other hand, gene research may lead to gene doping consisting of transduction of genes whose expression would enhance athletic performance.

Detection

Growth hormone (GH)

Growth hormone (GH), which is a strong anabolic substance, was included in the Prohibited List by the IOC, although no reliable GH detection method has been developed. The biggest problem with GH detection is the fact that GH is an endogenous substance which is very hard to differentiate from its exogenous form. The main form of somatothropin is a 22kDa isomer, however, due to the gene transcription and post-transcription modification new forms emerge. One of them is a 20kDa isomer, constituting 10% of the 22kDa isoform. Using this information Bidlingmaier et al. [27] developed a test detecting non-pituitary exogenous GH. The test used a mixture of nonspecific monoclonal antibodies and two specific monoclonal antibodies identifying epitopes of the 22kda and 20kDa isoforms. They showed that the absence of the 20kDa form in a blood sample containing a considerable quantity of the 22kDa isoform is evidence of an exogenous administration of rhGH.

The development of a test detecting rhGH requires establishment of the basic GH concentration norms. This could be very difficult as the GH is secreted by the pituitary gland in pulses in response to different kinds of external stimuli and changing physiological and biochemical status of the body. The GH concentration depends on:
- age,
- sex – women feature a higher GH concentration than men; this elevation can be caused by the use of oral contraceptives, different body composition, lower body mass and greater quantity of estrogens; the highest GH concentration is observed in women during the follicular phase of the menstrual cycle,
- "trainedness" status of the body,
- exercise – the blood concentration of GH may increase up to fifty times following intense physical exercise; this level is, however, maintained for about 60 min after the completion of the exercise [27].

A relationship was observed between the amount of released GH, applied training load, general efficiency and the type of sport. It should be remembered that in persons with an elevated basal GH, exercise does not stimulate the pituitary secretion of GH. A study of 96 athletes representing different sports revealed post-exercise elevated GH in middle-distance runners, rowers, swimmers and cyclists [28]. In defining the GH concentration norms it should be remembered that GH secretion can also be affected by such factors as diet, stress and exercise.

As GH features a high biological activity influencing a number of metabolic processes, a reasonable prospect may seem an analysis of substances directly dependent on GH, which can serve as GH markers. Certainly, only such markers must be identified whose concentration increases due to administration of considerable amounts of rhGH, but remains stable following intense exercise. This way the development of a drug test detecting exogenous GH might be possible [29]. There are two types of substances interacting with growth hormone:

(1) components of the GH/IGF-I axis, i.e. GH, IGF-I, and binding proteins of IGF-I:
- acid-labile subunit (ALS)
- IGFBP-2
- IGFBP-3

IGFBP-3 and ALS are modulators of IGF-I in blood serum; ALS increases the affinity between IGFBP-3 and IGF-I.

(2) markers of bone and collagen turnover:
- C-terminal cross-linked telopeptide of type I collagen (ICTP) responsible for bone resorption;
- amino-terminal propeptide of type III procollagen (PIIIP) responsible for formation of calluses;
- carboxyterminal propeptide of type I procollagen (PICP) responsible for bone remodeling;
- bone-specific alkaline phosphatase (BS-ALP) responsible for bone mineralization;
- osteocalcin responsible for bone mineralization.

A number of independent research teams have recently studied the effects of rhGH abuse and physical exercise on the blood concentration of the above markers in athletes representing various sports. The obtained results show that the level of these substances increases due to physical exercise but they feature a greater sensitivity to rhGh activity which enhances the effects of the exercise. The GH level rises following exercise and administration of rhGH, however, the level of the other components of the GH/IGF-I axis increases slightly and returns to its original value 30–60 min after exercise [30]. A higher concentration of IGF-I was only noted in the urine [30], despite the fact that physical exercise is known to induce the expression of the two main IGF-I isoforms, i.e. MGF and IGF-IEa) present in skeletal muscles. Also the level of markers of bone turnover increases insignificantly or remains unchanged, and returns to its original value or slightly decreases within 120 min following the completion of exercise. Only in the case of BS-ALP was a significant exercise-induced increase noted, and its concentration remained unchanged following an administration of rhGH [30]. It is commonly suggested that the blood concentration of these markers can be genetically determined [5].

The influence of rhGH on the markers of the GH/IGF-I axis and the markers of bone turnover is different: a significant increase of GH, IGF-I, IGFBP-3, PICP, PIIIP, ICTP, and a decrease of IGFBP-2 were noted. Also rhGH elevates the IGF-I/IGFBP2 ratio and lowers the IGFBP3/IGFBP2 ratio [30, 31]. Physical exercise in combination with rhGH administration was observed to enhance the response of the markers, whereas discontinuing rhGH administration inhibits the growth of GH, even in the same training conditions [29].

These results can be used in studies on development of drug tests. The markers discussed above feature a characteristic sensitivity to rhGH and a relatively poor response to physical exercise. A good chemical detector should be characterized by:
- stability in physiological conditions, during training, recovery period and after injuries;
- relatively long half-life.

The markers of bone and collagen turnover are present in the blood for at least 96 hours after administration of rhGH; some sources claim they can remain up to several weeks, i.e. for much longer than the markers of the GH/IGF-I axis.

In the process of development of effective drug tests the physiological norms of the markers must be established. They would then allow the assessment of GH blood and urine concentrations. An excessive level of GH concentration may be indicative of an administration of exogenous GH. However, the concentration of markers may depend on a number of different factors, e.g.
- type of sport,
- age,
- sex,
- place of origin,
- past diseases and injuries,
- taken medicines,
- disorders (undiagnosed acromegaly),
- training period (regeneration, preparatory, pre-competitive),
- marker's time of response to rhGH [29, 5].

No correlation has been found between the concentration of the studied markers and body height, body weight, BMI or VO_{2max}. The reference values of the markers have not been established yet, but the collected data was used in the international project GH-2000. The reference values of each marker were set for both sexes in conditions of maximal intensity exercise [29]. The changes in the level of a given marker during an athlete's annual training cycle can also yield important information.

The analysis of the markers of the GH/IGF-I and of bone and collagen turnover led to identification of two substances which can be very significant in development of GH abuse detection methods: PIIIP and ICTP. Their useful properties include:
- insignificant changes in response to intensive, long-term training;
- strong response to even most insignificant doses of rhGH;
- increased concentration up to 96 hours after discontinuation of rhGH administration, before and after exercise [29].

The main regulator of IGF-I synthesis in the liver is GH. IGF-I can thus act as a marker of GH activity in the liver and of the presence of rhGH in the body. Studies show that administration of rhGH significantly increases the blood level of IGF-I [10], and contributes to lower IGF-I secretion in the urine. This seems interesting since one of results of physical exercise is proteinuria, i.e. the presence of an excess of serum proteins in the urine. Studies of athletes in conditions of intensive exercise revealed an elevated post-exercise concentration of

IGF-I, creatinine, and protein in the urine [10]. The origin of post-exercise IGF-I in the urine is unknown; it may come from:

- circulation (directly),
- glomelural filtration rate,
- direct renal synthesis.

The identification of the IGF-I source and its normal concentration in the urine before and after exercise could be a basis for development of a test detecting rhGH abuse [10].

IGF I

An oral, intramuscular or intravenous administration of IGF-I significantly changes its blood and urine concentrations. The IGF-I levels can be easily established using common immunological tests. More difficult is the establishment of IGF-I physiological reference values in blood and urine. As mentioned earlier the IGF-I level varies, depending on a number of physiological and environmental factors. Its amount in blood and urine increases after exercise. Thus IGF-I concentration norms should be established before and after exercise at different intervals, as well as after taking exogenous IGF-I [10]. A reliable, positive drug test result is an individual matter. The IGF-I concentration in the blood and urine should be correlated with other physiological parameters, body weight, body height, intervals between exercises, diet and other factors affecting directly and indirectly the IGF-I concentration.

Equally difficult is the detection of a tranduced IGF-I gene inserted via a viral vector to the muscle tissue: the expressed gene is almost identical with the endogenous protein. The development of a drug test capable of identification the endogenous form from the exogenous one has so far proven unsuccessful. The difficulty in detecting IGF-I gene doping is also due to the fact that this protein is only produced in the target tissue in which the gene was expressed and it does not occur in the blood or urine. Detection of extra IGF-I molecules would be only possible by way of muscular biopsy [32].

Insulin

Detection of insulin abuse remains very difficult due to the impossibility of discrimination between its exogenous and endogenous forms [15].

EPO

The development of a drug test detecting rhEPO has been subject of intense research for several years. It will become possible after all differences between the endogenous and exogenous EPO forms are thoroughly analyzed. These differences are due to variable isolectric mobility resulting from different post-translational modifications of glycoprotein [18]. The main agents determining the differences are oligosaccharide molecules on the surface of erythropoietin, which participate in the plication and protection of the protein against proteases and influence its activity [16]. The synthesis of oligosaccharides as well as the saccharides of recombinant glycoproteins (rhEPO) is tissue- and cell-specific. Venke S. et al. [16] carried out a thorough analysis of endogenous and exogenous EPO forms and revealed that the altered structure of rhEPO is caused by the absence of tissue-specific synthesis, which does not take place in cell lines (due to lack of appropriate enzymes). Thus the two forms of EPO differ in type, structure and quantity of saccharide residues. Endogenous EPO contains more neutral sugars, whereas rhEPO contains numerous extra acidic saccharide residues which increase its molecular mass and extend the half-life of glycoprotein affecting its biological activity. The observed differences are also related to the sensitivity of both proteins to proteases [16]. The study focused on an analysis of serum EPO (sEPO), urinary EPO (uEPO), and rhEPO, revealing a lower molecular mass of sEPO than uEPO and rhEPO. Another study [16] proved that uEPO contained more acidic saccharides than sEPO. It indicates the existence of structural differences between the two forms, which can result from their different renal re-absorption patterns. Therefore a more detailed analysis of uEPO properties seems necessary as they may constitute a basis for detection of recombinant human erythropoietin doping.

Another promising study into detection of rhEPO abuse is concerned with the influence of rhEPO on the expression profile of erythrocyte marker genes: hemoglobine β (HBB), ferritin light chain (FTL) and ornithine decarboxylase antizyme (OAZ) (Tab. 2) [33].

An analysis carried out during rhEPO administration as well as three weeks after its discontinuation revealed an increasing level of expression of the aforementioned markers. As the effect of rhEPO activity is significantly shorter in the case of hematological changes as opposed to gene expression, it is indicated that

Table 2. Functions of erythrocyte markers

HBB (hemoglobin β)	FTL (ferritin light chain)	OAZ (ornithine decarboxylase antizyme)
Binding hemoglobin polypeptide chain	Storage of iron ions in a nontoxic state	Degradation of ornithine decarboxylase, control of the concentration of polyamines

these genes can serve as markers of rhEPO doping [33]. The detection of rhEPO in the body becomes increasingly difficult several days after discontinuation of its administration, even in case of taking small doses and enhancers of rhEPO activity (GH, IGF-I) [33].

Generally, rhEPO detection methods are classified as direct and indirect.

The direct methods, i.e. immunological tests, mark the concentration of EPO in the urine and blood serum. The main difficulty lies in discrimination between the exogenous and endogenous EPO forms and in detecting EPO only 2–3 days after the discontinuation of its administration. The immunological tests can detect immunoreactive protein forms but they are not always bioactive forms. Another difficulty in detection is a very short half-life of protein hormones, which makes it necessary to run drug tests in a very short period of time.

Functional studies [19] using electrophoretic and immunological analysis of EPO in the blood and urine revealed that the probability of rhEPO detection three days after rhEPO injection was less than 50%, and was almost impossible after 1–3 weeks. Some methods, e.g. High Performance Liquid Chromatography (HPLC) in combination with mass spectrometry and capillary electrophoresis, can yield interesting results but they require more detailed research.

Indirect methods of rhEPO detection include assessments of the following blood parameters:
• hematocrit
• hemoglobin (quantity),
• reticulocytes (quantity),
• erythrocytes (quantity, shape, size),
• serum transferrin receptor (sTfR),
as well as markers such as functional iron deficiency leading to the formation of hypochromic red cells. This deficiency is not neutralized, even if it is simultaneously supplemented with iron, and occurs immediately in response to rhEPO. It should be noted, however, that some athletes may feature elevated post-exercise HRC [19].

The use of hematocrit as a marker of rhEPO doping has brought negative results. Although its higher concentration might follow an administration of rhEPO, it depends on a variety of factors such as plasma volume, loss of fluids, dietary habits, training, iron metabolism, age, sex, body weight and circulating blood volume. The level of hematocrit also depends on the type of sport [19]. Overall, it is not a reliable detector of rhEPO abuse.

Also testing the quantity of reticulocytes does not yield satisfactory results either, due to the vast discrepancy between the reference values of their concentration [19].

The level the serum transferring receptor can be elevated in cases of:
• iron deficiency;
• increased demand for iron during stimulated erythopoesis;
• administration of rhEPO.

The sTfR was also regarded as a potential rhEPO marker, however, research results showed that it can act as a drug detector only in the case of administration of large doses of rhEPO without iron supplements [19].

Reliable rhEPO detectors were also sought among urinary markers such as fibrynogen degradation products (TDPs) resulting from the fibrinolytic activity of rhEPO. One study revealed the presence of TDPs in the urine of 10 out of 76 athletes. However, these results still require further analysis. There is no persuasive evidence either that an increased level of TDPs indicates the presence of rhEPO in the body [19].

The majority of researchers have focused on the impact of rhEPO on individual molecules being biochemical and hematological markers, and tried to determine their potential role in detection of rhEPO abuse. However, none of these markers can provide definite evidence of the presence of rhEPO in the human body.

In the late 1990s R. Parisotto et al. attempted to develop a drug test based on a few markers dependent on exogenous EPO. The subjects in their study were divided into three groups:
• subjects given an injection of EPO and iron supplements (injection);
• subjects given an injection of EPO and oral administration of iron supplements (stronger reaction to EPO);
• subjects given a placebo.

Each assessed parameter was assigned a specific number of points, averaged for each group. The differences between the groups provided with exogenous EPO and the control group were calculated. All possible combinations of parameters were estimated using the logit function. The developed test was based on 31 possible combinations of biochemical and hematological markers; it was approved by the IOC in July 2000 and used at the Summer Olympic Games in Sydney [34].

The rapid development of the pharmaceutical industry led to manufacturing of a synthetic form of erythropoietin called epoetin delta used to treat anemia associated with chronic renal failure. Standard urine rhEPO tests have all so far failed to detect it. Epoetin delta is synthesized in human cell lines and thus its properties are very similar to those of endogenous EPO. It can be detected with a blood test but the obtained result must be confirmed with a urine test [20].

It seems that a reliable method of detection of one of the most abused substances, rhEPO, has been developed. However, recent reports show that rhEPO doping still remains largely undetectable. Dr. Mike Ashenden, head of international consortium Science and Industry Against Blood Doping presented the most recent results of studies of cyclists taking part in Tour de France. These results show that in athletes who take microdose rhEPO regiments the concentration of hematocrit is elevated but only to an accepted level. Then a gradual reduction of the doses allows maintaining a high level of hematocrit. In this case a higher concentration of EPO is undetectable. Certainly, this kind of doping is possible under the supervision of a specialist who can administer the correct doses of the hormone [35].

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