**Biology of Sport, Vol. 24 N°4, 2007**

# HAZARDS ASSOCIATED WITH THE APPLICATION OF GENE THERAPY IN SPORT – GENE DOPING

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**Abstract.** The principal assumption of gene therapy is to achieve the therapeutic effect by a gene transfer into definite cells. It can be carried out on the entire organism (*in vivo* therapy) but also on collected cells, after their genetic modification, are returned back to the patient (*ex vivo* therapy). The therapeutic gene can be introduced using physical, chemical or biological methods. Vectors used in gene therapy are DNA molecules to which, by means of covalent bonds, a fragment of another DNA molecule can be joined and the entire unit can be introduced into the cell of the recipient. In the case of bacteria, plasmids or bacteriophages can serve as vectors, whereas in the case of eukaryotic cells, viral vectors are applied, e.g. papilloma, SV40 (papovaviruse), vaccinia virus, adenoviruses, retroviruses, baculloviruses etc. Genetic doping can be treated as a "splinter" of gene therapy. The prediction is that it is quite possible that on the next Olympic Games in 2008 the first genetically modified sportsmen can appear. Many investigations are focused on the detection of genetic "doping".

***(Biol.Sport 24:325-338, 2007)***

*Key words:* Gene therapy - Vectors - Genetic "doping" - Detection of genetic "doping"

## Background

According to the prevailing opinion in sports circles, it is quite possible that as early as during the next Olympic Games in 2008 in Beijing (Pekin), the first genetically modified sportsmen can appear. Is it possible that such fears are fuelled only by journalistic imagination and mass media? Alternatively, maybe, it is the

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realistic consequence of the introduction of genetic doping into sport arenas. In order to prepare properly to this potential threat, it is necessary to understand what, in fact, genetic doping is and what possibilities we have, here and now, of its detection. The present study makes an attempt to explain that, if certain specific conditions are fulfilled, there are real possibilities of detection and identification of genetic doping. Genetic doping is a "splinter" of gene therapy and, therefore, a considerable part of this article is devoted to it.

## Gene therapy

Gene therapy is a relatively young medical discipline established together with the development of molecular genetics. It arouses not only hope but also emotions as well as strong controversy. In addition, it also raises a number of doubts and questions and some of the principal issues, which are associated with this subject concern:

1. safety,
2. selection of diseases,
3. applied methods,
4. introduction of genes into cells,
5. effectiveness of gene transfer,
6. efficacy of the applied schedule of cell application containing the therapeutic gene.

The principal assumption of gene therapy is the following: to achieve the therapeutic effect by a gene transfer into definite cells.

A number of changes take place in the treated organism, some of which are considerably unfavourable. It may also happen that these unfavourable changes occur in genetic material and, consequently, the treated organism stops functioning properly and there are, practically speaking, no possibilities of the automatic and spontaneous repair of the damaged genetic material [7,14]. The developing product of the mutated gene may be either incorrect or may not develop at all, which means that gene expression does not take place. DNA damage may refer to a single or many genes.

The basis for the gene transfer success comprises:

- the transfer of the therapeutic gene into the target cell,
- achievement of its stable expression.

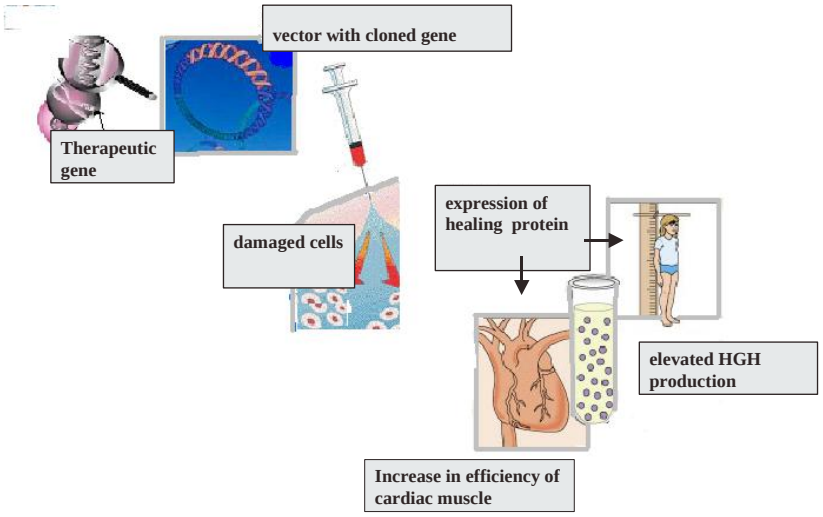


**Fig. 1**
An example of gene therapy application

Examples of diseases resulting from the damage (mutation) of one or many genes:

| Disease | Damage of a single gene | Damage of many genes |
|---|---|---|
| Mucoviscidosis | + | - |
| Haemophilia A and B | + | - |
| Severe combined immunodeficiency syndrome (SCIDS) | + | - |
| Duchenne muscle dystrophy | + | - |
| Tumours | - | + |
| Atherosclerosis | - | + |
| Rheumatic diseases | - | + |
| Parkinson's disease | - | + |
| Family hypercholesterolemia | - | + |

From the social point of view, gene therapy appears to raise the greatest hopes in such areas as cancer and AIDS treatments.

## History of gene therapy

The first successful experiment involving the transfer of the neomycin resistance gene into tumour infiltrating lymphocytes (TIL) was performed nearly 15 years ago. The first clinical attempt involving gene therapy was carried out on the 14$^{th}$ of September 1990 by Culver and his co-workers during an operation on Ashanti deSilve, a 4-year old girl suffering from the severe combined immunodeficiency syndrome (SCIDS), which is caused by the congenital deficiency of the adenosine deaminase (ADA) [10]. The applied treatment involved the transfer of the ADA gene into lymphocytes and, additionally, the patient was given the adenosine deaminase enzyme in the polyethylene glycol. In this case, the doctors applied the ex vitro therapy in which, first white blood cell were collected from the patient and proper copies of the damaged gene were introduced (transfected) into their nuclei and then the modified cells were re-introduced into the blood stream. The applied therapy resulted in the normalisation of the major parameters of the immunological response of both the cell and humoral types [3]. Understandably, the first successful application of the gene therapy significantly increased hopes and expectations among both patients and medical circles. However, it soon became clear that there was still a long way to go before widespread and successful application of gene therapy could be implemented in practice and considerable research in this field is still in progress. One of the disappointing examples could be the failed attempt of gene therapy carried out on the 18-year old Jessy Gelsinger who was suffering from a congenital malfunction of his liver from early childhood. The applied dose of virus turned out too high for the boy's immunological system and his organism began to battle not only with the virus but also with his liver, spleen and other internal organs leading to the death of the young man four days later. Nevertheless, approximately 3000 patents underwent some kind of gene therapy treatment during the period from 1990 to 2003.

## Ex vivo and in vivo therapy:

Gene therapy can be carried out on the entire organism of the patient (in vivo therapy) but also on cells collected from him/her [3,15], which, after their genetic

modification, are returned back to the patient (ex vivo therapy) [17]. The technique can be compared to the auto-transplantation of altered (transfected) cells.

| Trait | *Ex vivo* therapy | *In vivo* therapy |
|---|---|---|
| Efficacy | High | Unknown |
| Length of survival and functioning of genetically altered cells | Unknown | Unknown |
| *In vitro* cell culture | - | + |
| Gene transfer effectiveness | High | Low |

**Introduction of the therapeutic gene**

The technique of the DNA introduction is chosen in relation to the type of the transfected cells, the form of DNA and the expected form of expression. The therapeutic gene can be introduced using physical, chemical or biological methods.

Physical methods rely on the mechanical disruption of the continuity of the cell membranes of the target cells or amplification of their permeability by means of regulating the amount and size of pores. The simplest method of gene introduction is the injection of "naked" DNA. It is true that the direct injection of the plasmid DNA into muscles results in the transgene expression, but this happens on a relatively low level (cells take up about 1% of the applied dose). Earlier administration of the hypertonic saccharose solution or compounds inducing muscle regeneration enhances the efficiency of this method. If the expression of the therapeutic gene is to occur in the entire organism, the plasmid DNA requires protection against enzymatic degradation, e.g. cationic liposomes or polymers poorly reacting with DNA, such as PVP (polyvinylpyrolidon), which increases the transfer of the gene in the intramuscular injection 7 times [23].

The ballistic method utilises special guns to shoot in gold bullets wrapped in molecules of plasmid DNA into the target tissue. Hence, it is possible to administer the gene directly into the cell nucleus or cytoplasm without risking enzymatic degeneration but, in this case, the penetration depth does not exceed 0.5 mm and the expression disappears after 60 days. This method is particularly effective in intradermal vaccinations [23].

The cell transformation due to the applied electroporation takes place thanks to short electrical impulses of varying intensity, which lead to the higher permeability of the cell membrane, opening of pores and the infiltration of the plasmid DNA into the cell cytoplasm in accordance with the concentration gradient. The electrical field facilitates the electrophoretic and electroosmotic transport of

charged molecules such as DNA, across the membrane. In the case of the in vivo electroporation of such tissue as skin, liver as well as muscles, the naked DNA plasmid is injected interstitially, while electrical impulses are introduced by means of a needle or an electrode. This treatment enhances gene expression up to 1000 times in comparison with the ordinary plasmid DNA injection without electroporation. The in vivo optimisation of gene expression is affected by electrical current parameters and they, in turn, depend on the target tissue and should be determined empirically. In the case of muscle tissue, a weak (100-400 V/cm) electric field is sufficient [30,32].

In additions, other non-invasive physical methods are applied in various gene therapies, among others, ultrasounds (1 min., 1 MHz, 25 W/cm$^2$), which can increase the expression level by about 10-15 times, electrosonoporation, infrared lasers (smaller tissue damage than in electroporation), strong magnetic field (plasmid on nanomolecules) [30].

The chemical methods of the introduction of DNA into cells consist in facilitating its permeation through the cell membrane by means of endocytosis. For this purpose, crystals of calcium phosphate in a complex with DNA or DEAE-dextran are used (incubation with transfected cells in the culture medium with introduced DNA), which stimulate non-specific endocytosis and counterbalance the negative DNA charge. These methods are applied to study temporary expression in vivo [32].

Biological methods employ: viral vectors, i.e. appropriately modified viruses, which lost their reproductive capabilities in the cell but penetrate it easily and, once inside, they interfere with the host genome and allow a temporary gene expression or restitution as well as non-viral vectors, or the so called vectorless forms of DNA. They include oligonucleotides or DNA fragments with promoters and regulatory sequences. The place of the integration of such constructs is accidental. Oligonucleotides are introduced into cells by way of diffusion via neutral pores of the cell wall following their chemical modification protecting them against degradation.

Still another method of DNA protection against degradation and, at the same time, facilitating its transfer through the cell membrane is lipofection. Negatively charged DNA is neutralised by closing it in a positively charged lipid compound (lipofectins), which forms liposomes in aqueous environment. The addition to the cell culture of complexes: DNA – liposome results in a spontaneous merger of the liposome with the cell membrane and the liberation of DNA into the cytoplasm. Lipofection does not limit the length of the introduced DNA fragment – as in the

case of viruses – but it does not distinguish between cell membranes of different cells (is non-specific) [32].

## Vectors

Vectors are DNA molecules to which, by means of covalent bonds, a fragment of another DNA molecule can be joined and the entire unit can be introduced into the cell of the recipient. In the case of bacteria, plasmids or bacteriophages can serve as vectors, whereas in the case of eukaryotic cells, viral vectors are applied, e.g. papilloma, SV40 (papovaviruse), vaccinia virus, adenoviruses, retroviruses, baculloviruses etc. Vector forms of DNA allow accomplishing temporary expression of the introduced gene, the "knock out" effect or the restitution of the gene thanks to various regulatory sequences placed in the vector (e.g. promoter, enhancing transcription) and watching this effect thanks to reporter sequences.

The most common carriers of therapeutic genes are retroviruses, which are RNA viruses utilising, in the course of their life cycle, reverse transcriptase, which allows transcribing the RNA genome onto DNA. The viral DNA integrates with the host genome both in cells, which divide themselves [27], and in those which do not undergo divisions [16] and then it undergoes replication together with the host genes. Retroviruses are strongly unstable and, therefore, are used mainly in *ex vivo* therapy.

The integration mechanism of the virus with the host genome has not been fully recognised yet. However, it emphasises the fact that the random integration with the host genome can have influence on differences in the level of the transgene expression in different cells as well as on the transgene stability itself. The involved high risk concerns the fact that the random incorporation with the host genome may result in a malfunction of some genes, in particular those, which are situated in the incorporation region of the transgene, which may lead to mutagenesis. Nevertheless, the persistent expression, a simple genome easy for rearrangements as well as the relatively big capacity of the capsid (of the order of 10 kb (of the order of 10 kb) both affect the observed considerable application of retroviruses in gene therapy [25,31].

On the other hand, adenoviruses (circular viruses infecting many species of mammals, of which some are oncogenic viruses), due to their strong tropism to epithelial cells as well as to muscle and neuron cells, are used on a large scale mainly in the therapy of the breathing system, e.g., mucoviscidosis [2,9]. When used in the muscle gene therapy, they are applied, primarily, because of their efficient infusion into muscle cells, ease of propagation in large quantities, a

relatively high capsid content of the order of 8 kb, infection of non-dividing cells as well as the high level of expression [11]. One of the biggest limitations of the application of retroviruses is the strong immunological response of the organism they invoke directed not only against the virus itself but also against cells infected with the virus, which can lead to the damage of transduced cells. A mild immunological response was observed only in young individuals with a poorly developed immunological system [28]. For this reason, adenoviral vectors are modified by removing from them genes, which code strongly immunogenic proteins. In addition, adenoviruses are characterised by a complicated genome, which does not integrate with the host genome and the obtained expression is temporary and persists only for one year [25]. The temporary character of the transgene expression in the adenoviral vector makes them more useful in tumour treatments, where a long-term expression of the therapeutic gene is not necessary.

**Table 1**
Comparison of two main vectors applied in gene therapy

| Trait | Retroviruses | Adenoviruses |
|---|---|---|
| Stability | Low | High |
| Constraints | | Induction of the immunological response |
| Expression of the therapeutic gene | Persistent | Frequently temporary |
| Incorporation (incorporation into the patient's DNA | Frequently random | Is not incorporated into the patient's chromosomes |

The table below compares two of the most important vectors of therapeutic genes namely retroviruses and adenoviruses.

Promising investigations are being carried out employing adeno-associated viruses (AAV). They belong to a group of uncoated human viruses, whose genome is made up of a single-stranded DNA. A relatively small genome can contain the entire exogenous DNA of the magnitude of about 4.5 kb. AAVs accompany adeno- and herpesviruses in the course of infection, although they are not pathogenic themselves [25]. They exhibit very weak immunogenicity, which appears to be associated only with the presence of the transgene [31]. They infect a wide range of cells, both young and old, also those, which do not divide. They are capable of incorporating into the host genome, although a random place of incorporation can interfere with the proper functioning of cells [19]. These vectors were found to

survive in healthy muscles for a long time [25], which, when combined with their other advantages, including the ease with which these vectors can be prepared, encourages further investigations of the possibilities of AAV utilisation in muscle gene therapy.

## Expression regulation

Genes can undergo expression in almost any cell (the so called 'housekeeping' genes) or only in cells of specific tissues, in which case we talk about the tissue-specific expression. A similar situation occurs in the case of transgenes used in gene therapy. The introduced transgene can undergo expression either in all or only in certain cells. From this point of view, the control of gene expression of introduced genes becomes important. It can be regulated both non-specifically and specifically, depending on the presence of definite promoters or enhancers [24]. In the case of the non-specific expression, the housekeeping type elements ensure the expression in the majority of cells. In this case, the expression control, at this stage, is practically impossible and its magnitude will depend on: a/ the number of copies of the introduced therapeutic gene and b/ cell activity. Practically, it is possible to achieve the control over the expression regulation only in the result of a deliberate application of tissue-specific promoters, which will trigger off the expression of the introduced gene only in the target tissue without possibility of inactivation of the transgene in cells other than the target ones.

## Target cells in gene therapy

The following types of cells fall within the range of interest of gene therapy: glial cells, astrocytes, oligodendrocytes, Schwann cells, neuronal embryo cells, fibroblasts, mioblasts [4,13]. There is no doubt that, in comparison with the patient who is suffering in the result of one defective gene, it is much more difficult to apply gene therapy in patients who are suffering from diseases in which many genes are damaged, as is the case in tumours.

The main hazard for the organism concerns the fact that if a gene, for example one causing the growth of skeletal muscles, reaches the heart muscle, it will cause its hypertrophy. This, in turn, may result even in the death of the organism. Such dangers are numerous and it is necessary to speak loud about them, especially now, in the period when genetic doping is becoming increasingly popular among sportsmen and sportswomen.

## Genetic doping

A bizarre competition has been going on for years now between sportsmen using doping and scientists trying to expose this practice in order to allow young men and women to compete with one another along clean and clear sporting criteria. Scientists' efforts aiming at the identification of banned doping compounds in sportsmen's organisms resulted in a considerable expansion of possibilities of analytical investigations. Recent technological advances, amongst others, the development of liquid and gas chromatography, have significantly contributed to the fact that the number of the so called undetectable doping agents has been dramatically reduced [8]. There is far more information indicating that the known doping compounds harmful to health and easy to detect are being abandoned. They will be probably replaced by genetic doping, which is far more effective but more difficult to detect.

Genetic engineering is a branch of science, which has been developing dynamically in recent years. It is generally believed that in several years, scientists will be in a position to interfere in genes at will. Gene therapy will be applied in the treatment of a number of diseases, prevention of aging processes and also, quite probably, in sport.

Sportsmen and sportswomen have already successfully tried out new drugs, which could be useful in breaking records. This is how erythropoietin (EPO) found its way into sport and became the most popular doping agent of 1990s. This is a hormonal drug manufactured biotechnologically and useful in the treatment of anaemia, cancer, AIDS. It improves the supply of various organs in oxygen, which is achieved by increasing the bulk of erythrocytes; hence, its use also improves physical fitness [12,18]. The effect of erythropoietin administration appears already after 3 days and persists for up to 4 weeks. The administration of the EPO in cases therapeutically unwarranted poses a serious hazard to health and even life. The increase of the hematocrit results in the increase of blood viscosity, which slows down the blood flow, creates conditions for the development of intravascular clots, and may also lead to the increase of blood pressure. This results in disturbances of the blood supply to the brain. The developing thrombin can lead to pulmonary embolisms, cardiac infarctions or cerebral strokes. It appears that sportsmen are especially exposed to the occurrence of this type of reaction following erythropoietin administration because, following a long-term effort, their blood has already undergone thickening in the result of water loss [29]. The application of erythropoietin has already resulted in a number of deaths, especially among cyclists.

For a long time, the EPO has been difficult to detect, using traditional anti-doping tests because, after its injection, its level goes back to normal very rapidly (4 to 7 days). However, at the present time, there are no longer obstacles to detect it in blood or urine. Therefore, for sportsmen who want to break records or reach the best sport results, it will be an ideal solution if stimulating substances were manufactured in their organisms themselves.

Recently scientists located genes responsible for the production of erythropoietin. Therefore, it will soon be possible to enhance the efficiency of the human organism in the result of genetic modification. The effectiveness of this kind of manipulation has already been confirmed on animals. It was found that the increased quantity of red blood cells remained unchanged for 20-30 weeks following the introduction into monkey muscles of the gene responsible for the production of erythropoietin [26]. However, it should be remembered that the introduced gene can undergo an uncontrolled expression and the organism will begin manufacturing excessive quantities of red blood cells, which may result in fatal complications.

Investigations are also being conducted which employ various growth factors, such as: the growth hormone, insulin-like growth factor (IGF-1) as well as vascular endothelium growth factor (VEGF). The applied growth hormone can affect tissues either directly or indirectly. The direct influence occurs by the stimulation of the production of the insulin-like growth factor IGF-1 and IGF-2. These factors are synthesised in the liver and other tissues. The IGF-1 enhances the transport of amino acids to cells and protein synthesis, consequently leads to the increase of muscle weight. The direct influence of the growth hormone includes: the fat tissue in which it increases lipolysis, the muscle of skeletal muscles where it inhibits glucose transport into cells and the liver in which it intensifies glucose production. The secretion of the growth hormone changes in individual periods of our lives. Its highest values are achieved during the period of adolescence, while in adult individuals, the secretion of this hormone decreases gradually [6].

Scientists from a University in Pennsylvania, administered mice the gene that codes the insulin-like growth factor IGF-1. They achieved it with the assistance of an adenovirus penetrating into the muscle cell cytoplasm. It was found that the increase of muscles in the feet of young individuals improved by 15-20%, which was not observed in adult individuals. It should be stressed, however, that the muscle weight in the latter group stopped diminishing. With the passage of time, it was greater than in mice from the same age group, but which were not given the IGF-1 gene [1].

The physical effort capacity of sportsmen depends, among others, on the supply of oxygen to muscles. Improved vascularisation of muscles is of significant physiological importance as a factor, which increases the oxygen consumption by muscles. In addition, this also leads to the increased availability of energetic substrates. It was demonstrated that several-week long endurance training increased the amount of capillaries in muscle even by 30 to 40% [5]. However, once the training is discontinued, this condition persists only for a few weeks.

Similar effects can be achieved following the transfer of the VEGF by a virus. These types of investigations were performed for therapeutic purposes on patients after cardiac infarctions and suffering from the atherosclerosis of peripheral vessels. The transfer of the gene resulted in the intensification of angiogenesis. However, fears have been expressed that vessels will develop without any clear purpose. The administration of the growth factor may result in the development of a tumour because cells, which normally would not divide, begin to do so.

Results of scientific research indicate that it will soon be possible to genetically tailor, customise or condition a sportsman/sportswoman to the sport discipline he or she goes in for (World of Science). Basically, there are two kinds of cells in human muscles: rapidly- contracting ones (responsible for speed) and slowly contractile (affecting endurance). The speed of development of the maximum muscle stress is determined by the type of myosin. Nuclei of muscle cells contain the genetic material, which allows synthesising any kind of myosin and, whether the rapidly- or slowly-contracting muscle cell is actually developed depends on which type of gene expression actually takes place.

The performed studies indicate that sprinters have a different setup of gene than long-distance runners. In addition, in sprinters researchers found larger quantities of the enzyme called angiotensin convertase – ACE [21,22]. Scientists have successfully isolated the gene responsible for the synthesis of ACE. Therefore, it can be assumed that following the introduction of these genes, it will be possible to alter the composition of muscles and to manufacture any quantity of rapid or slow fibres [20]. All this has been made possible thanks to gene therapy and methods worked out in the course of experiments on mice, rats or other animals. Unfortunately, it is to be feared that the application of genetic doping will also allow “breeding” of genetically improved sports-men and women with whom sportsmen and sportswomen who refuse to undergo genetic modification will find difficult to compete.

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Accepted for publication 7.07.2005