UNIVERSIDADE FEDERAL DE PELOTAS
Programa de Pós-Graduação em Biotecnologia

DISSERTAÇÃO

Efeito da administração de eritropoetina e de vetores recombinantes em parâmetros reprodutivos de coelhos

Thaís Farias Collares

Pelotas, 2010.

THAÍS FARIAS COLLARES

Efeito da administração de eritropoetina e de vetores recombinantes em parâmetros reprodutivos de coelhos

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Orientador: Prof. João Carlos Deschamps, PhD.

Comissão de Orientação: Prof. Fabiana Seixas, Dra.

Prof. Odir Dellagostin, Dr

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Prof. Dr. João Carlos Deschamps, Universidade Federal de Pelotas

Prof. Dra. Fabiana Kömmiling Seixas, Universidade Federal de Pelotas

Prof. Dra. Marta Gonçalves Amaral, Universidade Federal de Pelotas

Prof. Dra. Cláudia Pinho Hartleben, Universidade Federal de Pelotas

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RESUMO

COLLARES, Thaís Farias. **Efeito da administração de eritropoetina e de vetores recombinantes em parâmetros reprodutivos de coelhos**. 2010. 51f. Dissertação (Mestrado) – Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas. Pelotas.

A administração de proteínas recombinantes vem sendo utilizada no esporte como um meio de doping. Na medicina, um método terapêutico bastante recente é a terapia gênica, que até o momento, possui resultados indicando sua eficiência no tratamento de algumas doenças. Recentemente, o potencial para uso indevido desta terapia entre atletas tem despertado a atenção de cientistas e órgãos reguladores do esporte. A transferência de genes que poderiam melhorar o desempenho esportivo de atletas saudáveis foi denominada de doping genético. Os principais genes candidatos são eritropoetina (EPO), fator de crescimento endotelial vascular (VEGF), fator de crescimento semelhante à insulina tipo 1 (IGF-1) e bloqueadores da miostatina. Porém a terapia gênica apresenta indicadores adversos, como resposta inflamatória e falta de controle da ativação do gene. Em indivíduos saudáveis, é provável que essa situação seja agravada. Ainda não existem testes conclusivos para a detecção do doping genético, no entanto alguns estudos recentes têm o intuito de investigar algumas estratégias que apontam como promissoras. O reflexo do uso da EPO sobre parâmetros reprodutivos *in vivo* ainda não tem sido descritos, por outro lado, estudos *in vitro* com cultivo de células têm demonstrado que a eritropoetina estimula a esteroidogênese nas células de Leydig desencadeando um aumento na produção de testosterona. O objetivo deste estudo foi avaliar os efeitos da administração de rHuEpo (eritropoetina recombinante) e da transferência gênica com eritropoetina em parâmetros reprodutivos de coelhos. Quinze coelhos foram divididos em 3 grupos: grupo I (rHuEpo) receberam por via subcutânea 25UI/kg de eritropoetina humana recombinante, três vezes por semana durante cinco semanas, grupo II (pTarget/Epo) receberam dose única de vetor recombinante com o gene da eritropoetina de coelho; grupo III (pTarget) receberam dose única de vetor pTarget vazio (controle). Parâmetros sanguíneos e reprodutivos foram monitorados durante o experimento, tais como: motilidade, vigor espermático, concentração espermática, viabilidade espermática, morfologia espermática, hemácias e hematócrito. A

transferência gênica com eritropoietina e a administração de rHuEpo causaram um aumento significativo no número de eritrócitos. Os animais que receberam rHuEpo obtiveram um aumento no hematócrito, alcançando valores entre 41,34 e 52,32. A análise estatística mostrou que os tratamentos e o tempo não interferiram na motilidade, concentração espermática e vigor espermático (P <0,05). A porcentagem de células com morfologia normal, tanto do grupo I como do grupo II diminuiu no decorrer do tempo, mas não houve diferença estatística entre os tratamentos (P<0,05). Este é o primeiro estudo que relata as respostas do uso do doping genético com o gene da eritropoetina e da administração de rHuEpo em parâmetros sanguíneos e reprodutivos.

Palavras-chave: doping genético; rHuEpo; terapia gênica; parâmetros reprodutivos

ABSTRACT

COLLARES, Thaís Farias. **Efeito da administração de eritropoetina e de vetores recombinantes em parâmetros reprodutivos de coelhos**. 2010. 51f. Dissertação (Mestrado) – Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas. Pelotas.

The administration of recombinant proteins is being used in sport as gene doping. In medicine, a recent therapeutic technique is the genetic therapy, which, up to this moment, shows results that indicate its efficiency in the treatment of some diseases. Recently, the potential for misuse of gene therapy among athletes has called the attention of scientists and sports regulating organs. The transfer of genes that could enhance athletic performance was named gene doping. The most important candidate genes for gene doping are Erythropoietin (EPO), vascular endothelial growth factor (VEGF), insulin-like growth factor I (IGF-1) and myostatin blockers. Nevertheless, gene therapy presents adverse indicators, such as inflammatory response and lack of control of gene activation. It is probable that in healthy individuals such problems would be aggravated. There are still no conclusive tests capable of detecting gene doping. However, recent researches have studied promising strategies. The reflection of the use of EPO on reproductive parameters *in vivo* has not yet been described. On the other hand, *in vitro* studies with cultured cells have shown that erythropoietin stimulates steroidogenesis in Leydig cells, triggering an increase in testosterone production. The objective of this study is to evaluate the effects of the administration of recombinant erythropoietin (rHuEpo) and erythropoietin gene transfer in reproductive parameters of rabbits. Fifteen rabbits were divided in 3 groups: group I (rHuEpo) received subcutaneously 25UI/kg of recombinant human erythropoietin, three times a week for 5 weeks; group II (pTarget/Epo) received a single dose of recombinant vector with the gene of the rabbit erythropoietin; group III (pTarget) received a single dose of empty pTarget vector (control). Throughout the experiment, reproductive and blood parameters were monitored, such as: sperm motility, spermatic vigor, sperm concentration, sperm viability, sperm morphology, erythrocytes and hematocrit level. Erythropoietin gene transfer and rHuEpo administration caused a significant increase in the number of erythrocytes. The animals which received rHuEpo showed an increase in the

hematocrit level, reaching numbers between 41,34 and 52,32. The statistical analysis proved that the treatment and the time did not interfer on sperm motility, sperm concentration and spermatic vigor ($P<0.05$). The percentage of morphologically normal cells in group I as well as in group II decreased over time, however, there was no statistical difference between the treatments ($P<0.05$). This study is the first to show the answers to the use of gene doping with the erythropoietin gene and the rHuEpo administration in reproductive and blood parameters.

Key words: gene doping; rHuEpo; gene therapy; reproductive parameters

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LISTA DE ABREVIATURAS E SIGLAS

ANOVA – Análise de Variância
AZT – Zidovudina
cDNA – Ácido Desoxirribonucléico Complementar
DNA – Ácido Desoxirribonucléico
EPO – Eritropoetina
GH – Hormônio do crescimento
GnRH – Hormônio liberador de gonadotrofina
HIV – Vírus da Imunodeficiência Humana
IGF-1 - Fator de crescimento semelhante à insulina tipo 1
LH – Hormônio Luteinizante
mRNA – Ácido Ribonucléico mensageiro
rHuEPO – Eritropoetina Humana Recombinante
VEGF - Fator de crescimento vascular endotelial

SUMÁRIO

1. INTRODUÇÃO GERAL

A eritropoetina (EPO) é o regulador primário da eritropoiese, a qual é um hormônio glicoprotéico produzido inicialmente no fígado de fetos e mais tarde nos rins. Este hormônio age sinergicamente com outras citocinas para promover a proliferação, diferenciação e sobrevivência de células progenitoras de linhagens eritróides (VARLET-MARIE et al., 2004).

A rHuEPO (eritropoetina recombinante humana) é aprovada para uso em humanos no tratamento de anemias, associada com hemodiálise ou doença renal crônica, pacientes com câncer em quimioterapia, pacientes HIV positivos em terapia com AZT, bem como para minimizar o uso de transfusões de sangue alogênica em pacientes anêmicos submetidos a grandes cirurgias (LANGSTON et al., 2003).

Nenhum produto derivado da eritropoetina está aprovado para uso em animais, no entanto, a eritropoetina recombinante humana é comumente utilizada no tratamento da anemia promovida por falha renal crônica. O risco de abuso da eritropoetina recombinante é aumentado pela sua obtenção e administração sem supervisão médica (SHARPE et al., 2002).

Em esportes de resistência e corridas de cavalo, especula-se que a rHuEPO passou a ser utilizada de forma rotineira como meio artificial de produção de glóbulos vermelhos, devido a vantagem adicional da difícil detecção de sua presença na matriz biológica através dos métodos analíticos convencionais, além do efetivo ganho no desempenho esportivo (GUAN et al., 2007; PASCUAL et al., 2004).

O uso de eritropoetina recombinante para aumento de performance de atletas é ilegal, mas a prática presumivelmente continua ilícita (ASHENDEN et al., 2006). Em cavalos de corrida, qualquer aumento de performance promovido pela EPO é obscurecido pelo risco de formação de anticorpos contra as moléculas administradas e com possível reação contra moléculas de eritropoetina endógena. Observações

preliminares sugerem que o uso abusivo de eritropoetina recombinante pode provocar um risco de parar abruptamente a produção de eritropoetina endógena, desencadeando uma severa anemia. Nesse caso, estes indivíduos seriam incapazes de desenvolver respostas eritropoiéticas adequadas a condições de estresse (AZZAZY et al., 2005).

Efeitos adversos do uso da eritropoetina recombinante são anemia refratária/hipoplasia eritrocitária, hipertensão sistêmica, policitemia, ataque apoplético, vômito, deficiência de ferro, reações na pele, febre e artralgia. Hipertensão também tem sido associada à terapia com eritropoetina em pessoas e animais (LANGSTON et al., 2003). A eritropoetina também aumenta notavelmente a ativação endotelial e a reatividade plaquetária em humanos, e isto pode aumentar substancialmente o risco de complicações tromboembólicas, especialmente em indivíduos com predisposição genética à trombofilia (CAZZOLA, 2000; DIAMANTI-KANDARAKIS et al., 2005; LASNE et al., 2004).

Em geral, sabe-se pouco sobre os efeitos do tratamento com rHuEPO por um longo período com fatores de crescimento hematopoiéticos, mas observações em animais sugerem que há potencialmente um risco de desenvolvimento de desordens mieloproliferativas (CHENUAUD et al., 2004).

Experimentalmente, o gene que codifica a eritropoetina vem sendo administrado no tratamento de anemias e de doenças renais, uma vez que produz uma forma de hormônio efetivamente endógena. Esta ferramenta de terapia gênica pode ser utilizada como doping genético (LASNE et al., 2004).

O termo doping genético tem origem na utilização de proteínas recombinantes e vetores de DNA para aumentar o desempenho atlético em humanos e animais. (BAOUTINA et al., 2008). O doping genético é baseado na introdução e subsequente expressão de um gene alvo ou por modulação da atividade de um gene existente.

Por exemplo, o doping genético, com IGF-1 tem sido bem sucedido em ratos, onde um aumento perceptível na massa e força muscular foi observado mesmo meses após o tratamento concluído (BARTON-DAVIS, 1998). O doping utilizando a EPO demonstrou efeitos sistêmicos em macacos, incluindo o aumento da capacidade aeróbia, a melhoria do desempenho e os níveis de hematócrito elevado (UNAL et al., 2004). Porém alguns estudos relataram um aumento de hiperatividade, agressividade e outras sequelas comportamentais nos ratos tratados. A superexpressão de EPO em macacos tem sido relatada por aumentar a viscosidade

do sangue, com efeitos sobre o funcionamento cardíaco (MCKANNA, 2010). É evidente que, embora modelos animais possam demonstrar a promessa do doping genético, os perigos desse procedimento não podem ser ignorados, porque a sua utilização está prevista em seres humanos.

Ainda não existem testes conclusivos para a detecção do doping genético, no entanto alguns estudos recentes têm o intuito de investigar algumas estratégias que apontam como promissoras (DEFRANCESCO, 2004; HAISMA et al., 2006; UNAL et al., 2004).

Qualquer processo fisiológico envolvido na produção de uma ação motora ou de auxiliar na execução de um movimento motor pode ser um candidato para o doping genético. Os processos fisiológicos da respiração pulmonar, circulação cardiovascular, de fornecimento de oxigênio, o crescimento do músculo estriado/eficiência/reparação, e até mesmo a coordenação neuromuscular podem ser alterados para dar ao atleta uma vantagem na sua competição. Alguns genes são candidatos potenciais para uso em doping genético como: GH, IGF-1, VEGF, EPO e Miostatina, os quais também poderão ser utilizados para promover a cura mais rápida de lesões esportivas, e reduzir dor associada (BAOUTINA et al., 2007; GAFFNEY et al., 2007).

Embora o uso adequado destes hormônios traga inúmeros benefícios terapêuticos no tratamento de certas doenças, o seu uso impróprio pode traduzir-se em enormes riscos para os atletas que utilizam para ganhar vantagem em termos competitivos.

Com base nos atuais conhecimentos sobre respostas do organismo, celular e sistêmica, gerados a partir da engenharia genética, imunologia, bioquímica e fisiologia busca-se uma compreensão dos efeitos e conseqüências sobre o metabolismo de novos peptídeos gerados por processos biotecnológicos.

O reflexo do uso da EPO sobre parâmetros reprodutivos *in vivo* ainda não tem sido descritos, por outro lado, estudos *in vitro* com cultivo de células têm demonstrado que a eritropoetina estimula a esteroidogênese nas células de Leydig desencadeando um aumento na produção de testosterona (YAMAZAKI et al., 2004). Essa observação leva a formulação da hipótese de que a administração de rHuEPo *in vivo* induz ao aumento na síntese de testosterona levando a um feedback negativo sobre a liberação de GnRH/LH e conseqüentemente afetando direta e indiretamente

a espermatogênese e o potencial espermático podendo desencadear uma infertilidade temporária ou permanente.

As metodologias apresentadas, utilizando modelos biológicos experimentais, buscam a compreensão das conseqüências fisiológicas da administração do gene da eritropoetina humana.

Inicialmente é apresentada uma revisão bibliográfica (artigo I) sobre o uso de genes para o aumento da performance atlética, onde foi relacionado o doping genético e a terapia gênica, intitulada: “The use of genes for performance enhancement: doping or therapy? Esta revisão foi submetida à publicação na revista Sports Medicine.

Em seguida, o artigo II intitulado: "Effect of rHuEpo and erythropoietin gene transfer on reproductive parameters of rabbits", onde o objetivo foi avaliar o grau de comprometimento do potencial reprodutivo dos modelos biológicos submetidos a administrações de eritropoetina recombinante ou de vetores de DNA contendo o gene da eritropoetina. Este trabalho será submetido à publicação, como artigo completo, para a revista Scandinavian Journal of Medicine & Science in Sports.

Os dados encontrados estão apresentados na forma de artigos científicos logo a seguir. Os artigos estão compilados na formatação exigida por cada um dos periódicos científicos em que foram ou serão submetidos.

ARTIGO I

The use of genes for performance enhancement: doping or therapy?

(Artigo científico formatado nas normas do periódico *Sports Medicine*)

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The use of genes for performance enhancement: doping or therapy?

Thaís Farias Collares, Ruan Oliveira, Vinicius Farias Campos, Priscila Moura Marques de Leon, Fabiana Kömmiling Seixas, João Carlos Deschamps and Tiago Collares*

Centro de Desenvolvimento Tecnológico (CDTec), Programa de Pós-Graduação em Biotecnologia – PPGB, Universidade Federal de Pelotas, Pelotas, RS, Brazil.

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***Corresponding Author**
Tiago Collares
Centro de Desenvolvimento Tecnológico
Universidade Federal de Pelotas
Campus Universitário s/nº, CEP 96010-900
Caixa Postal 354, Pelotas, RS, Brazil
Phone: +55 53 3275 7588, FAX: +55 3275 7551
E-mail: collares.t@gmail.com

Abstract

Recent advances in biotechnology have provided the manipulation of genes to treat various pathologies in a process called gene therapy. However, the improvement of this therapy and the potential to enhance athletic performance has opened the door for gene doping. In this technique, genes are inserted into a specific tissue, altering the gene expression or the amount of a protein product. The commonly used genes are erythropoietin (EPO), vascular endothelial growth factor (VEGF), insulin-like growth factor 1 (IGF-1) or myostatin antagonists, but a lot of new genes might be used, such as those involved in metabolic pathways of glucose. Because it is very difficult to detect, gene doping is the most promising method to corrupt the sports. Unfortunately, the scientific progress may enable new methods of doping. Thus, it is necessary to continually explore new ways of detection, allowing the return of sports to its original purpose, the achievement of recognition and glory.

Key-words: Gene doping, gene therapy, bioethics.

1. Introduction

The scientific knowledge has made many advances in recent years. In the last decade, the publication of the human genome, allied with various molecular knowledge existing at the time, provided the gene manipulation for the treatment of various diseases. The advent of molecular sciences also provided interventions in sports performance. Around the world, people have been exposed to the notion of human enhancement through sport, as some athletes seek a boost to success, stardom, and financial reward. In the past, doping and cheating in sport have been enabled by advances in pharmacology and physiology. Recently, the successful development of gene therapy has provided the concepts, tools, opportunities, and, for some, justification for genetic modification of functions that affect normal human traits, including athletic performance[1].

Gene expression is regulated primarily by two main mechanisms: (a) changes in the DNA structure or (b) direct interventions in the process of transcription and translation. The first includes the epigenetic modifications and mutations. By epigenetics, the availability of gene transcription is altered, without causing changes in DNA sequence. The mutations promote changes in the nucleotide sequence of the gene and can unchain the process of transcription or generate a new product, different from the original. The second mechanism consists of repressor and inductor molecules, transcription factors, enhancers and post-transcriptional modifiers. Therefore, some changes in the regulatory process may result in an increased or decreased concentration of the gene product.

The World Anti-Doping Agency (WADA; 2010 prohibited list) considers gene doping as the use of pharmacological or biological agents that alter gene expression, or the transfer of cells or genetic elements (DNA and RNA)[2]. As explained by N.C. Craig Sharp, some changes in gene expression are convenient to increase sports performance by many ways. These changes can target a wide variety of tissues, such as bone marrow for increase erythrocytes and enhance the oxygen transfer; liver and kidney for Cori-cycle lactate-removing kinetics; myocardium for increased cardiac output; and skeletal muscle to influence fiber-type quality and percentage, sarcomere structure, creatine-phosphate level, mitochondrial number, glycolytic and glycogenesis enzymes and chemiosmotic pathway kinetics, levels of myoglobin and

intracellular dipeptide buffers, and muscle capillary numbers. Also, the neurologic areas may be targeted to increase pain tolerance[3].

The fast advancement of biotechnology promotes the development of doping. From recombinant protein to gene doping, there is a great challenge to their detection[4]. The new opportunities generated by recent scientific advances require a thorough ethical analysis. What is the limit to reach victory? The abuse of gene therapy should be ignored? Is science destroying sports?

2. The Bioethical Analysis of Gene Doping

The real reason for the participation of athletes in sports competitions has changed radically since its creation. In the Ancient Olympic Games, athletes competed for recognition, eternal fame and an olive branch. Today, the participation in international competitions has another motive: money. For an athlete, to win a medal is a guarantee of lucrative contracts in the future[5].

Dr. Andy Miah explains that there are two ways to analyze the ethic acceptability of gene doping. The first believes that sports ethics is subservient to medical ethics. So, if the use of gene therapy for medicine is allowed, it should also be acceptable to performance increases. This way, a sports physician can prescribe potentially performance-enhancing drugs to athletes in order to alleviate some illness. However, this matter is not straightforward and it is not an easy task for a physician to decide how best to treat their athlete-patient – more as an athlete or more as a patient. Furthermore, some patients might have more interests in receiving a treatment that makes them well for sports performance, rather than well for life. The second way says that sport's ethics is separated of medical ethics. Sport is a moral practice, not requiring an approach that rejects the literature of medical ethics, but depends more on the sport context than on the medical context [6].

The main argument used by WADA to justify the prohibition of gene doping consists on the uncertainty created by the insertion of genes or the use of substances that interfere in the gene expression. The use of such substances can bring unknown risks to the athlete's health which can interfere not only in somatic cells, but also in the germline. In contrast to somatic cell therapy, germ-line alterations are permanent and are transmitted to future generations. Moreover, the use of gene therapy in order to increase sports performance violates the sports spirit,

giving unfair physical advantages to those who have a greater access to substances used in the process [7].

3. Candidate Genes for Use in the Gene Doping

3.1 Molecular Mechanisms of Synthesis and Action of Erythropoietin

In adults, the erythropoietin is produced mostly by the interstitial cells of kidneys and in small quantities by the liver, where fetal synthesis occurs [8]. This process is regulated by the concentration of circulating oxygen, differing in the conditions of normoxia and hypoxia. The enzymes prolyl hydroxylase and asparaginyl hydroxylase are sensors of the level of intracellular oxygen. In normal oxygen tension (normoxia), the hypoxia-inducible transcription factor 1α (HIF-1α) has its proline residues 402 and 567 hydroxylated in sites of proteasomal degradation dependent of O_2. In sequence, occurs the hydroxylation of asparagine residues in carbon terminal transactivation domain (C-TAD). These hydroxylations prevent the binding of HIF-1α to the nucleotide sequence (A/G) CGTG, a regulatory region of the erythropoietin gene (EPO). However, the opposite occurs in conditions of hypoxia - the hydroxylations not occur, resulting in binding of HIF-1α to the regulatory region of the EPO gene. Thus, in hypoxia occurs in the induction of gene expression in question, leading to increased levels of erythropoietin intracellular. Other transcription factors are also involved in regulating gene EPO expression, such as HIF-1β, HIF-2α / β and HIF-3α / β. After the transcription and translation of the EPO gene, the polypeptide originated suffers post-translational modifications, such as glycosylations, essential for the establishment of its function *in vivo* [9;10].

The hematopoietic system is regulated by three main groups of hematopoietic growth factors: (1) the colony stimulating factors, (2) thrombopoietin and erythropoietin and (3) cytokines (mainly interleukins). The erythroid lineage, which ultimately originates the erythrocytes, is regulated primarily by signaling performed by erythropoietin. The erythropoesis occurs in several steps of cell differentiation: the stem cell originates the pluripotent hematopoietic myeloid progenitor that differentiates into the colony-forming unit erythroid (CFU-E). Erythropoietin stimulates the proliferation and differentiation of this CFU in basophilic, polychromatic and orthochromatic erythroblast, in this order. These last differentiate into reticulocytes.

Last, but not least, the reticulocytes mature and produce the erythrocytes, cells that contain hemoglobin, a protein involved in gas exchange during cellular respiration [8]. By these cells, oxygen is carried to tissues and, arriving at its destination, it is used primarily for energy production, through the oxidative phosphorylation. Therefore, an increase in the number of circulating red blood cells, leads to an increase in the supply of oxygen to tissues and, thereafter, to greater energy production by aerobic mechanisms. This is the basic principle for the use of EPO in gene doping.

In the bone marrow are the early stages of the CFU-E progeny. These have a dimmed receptor for erythropoietin, whose intracellular domain has a tyrosine kinase coupled, called JAK-2 (Janus Kinase 2). The binding of the erythropoietin to the receptor, induces the interaction of JAK-2 with the SH2 domain of cytosolic protein STAT 5 (signal transducer and activator of transcription 5). This interaction promotes the phosphorylation of STAT 5, forming a homodimer of phosphorylated STAT 5. This translocates to the nucleus, binds to specific nucleotide sequences in DNA and promotes the transcription of genes necessary for the continuity of erythropoiesis [10].

Erythropoietin has other physiological effects, non-hematopoietics, as neuroprotective actions [11], found in patients with schizophrenia by Ehrenreich et al. [12]. Although there are no descriptions of the reflections of EPO on the reproductive parameters *in vivo* and *in vitro*, studies with cell cultures have shown that erythropoietin stimulates steroidogenesis in Leydig cells, triggering an increase in testosterone production, leading to a negative feedback on the release of GnRH/LH and, consequently affecting the spermatogenesis and the spermatic potential that may cause infertility [13].

The recombinant human erythropoietin (rhEPO) is produced in large scale by biotechnological processes and has wide application for the treatment of various pathologies, such as anemia, chronic renal insufficiency, hematological malignancies, chemotherapy and premature birth. Also, rhEPO is used to minimise allogeneic blood transfusions after major surgical procedures and recently for performance enhancement [14].

Fattori et al. analyzed the efficacy of intramuscular injection of the EPO gene along with the application of electric pulses to optimize the process of transduction. The gene was electro-injected into mice, rabbits and cynomolgus monkeys to test for protein production and biological effect. The study concluded that the injected EPO gene yields higher levels of circulating transgene product and a more significant

biological effect than the wildtype gene in all the species tested, thus showing great potential in clinically developable gene therapy approaches for EPO delivery [15].

3.2 VEGF: Increasing the Supply of Oxygen to the Tissues

The oxygen is vital for the synthesis of ATP by aerobic respiration. This gas is a small molecule that can diffuse through the plasma membrane of endothelial cells. Therefore, an increased vascular branching promotes a more rapid and effective diffusion of oxygen to the tissues and a greater availability of it for energy production. The vascular endothelial growth factor (VEGF) promotes the branching of a preexisting vessel, in a process called angiogenesis. In gene doping, are inserted several copies of the gene coding for VEGF in the muscle, using viral vectors. Thus, the muscular microcirculation is stimulated and, with this, the supply of oxygen to the muscles is increased [16;17].

3.3 IGF-1: The Stimulant of Muscle and Bone Growth

The insulin-like growth factor type 1 (IGF-1) is a 70-amino-acid polypeptide synthesized primarily in the liver under the control of the growth hormone (GH) [17;18]. The hypothalamus produces two peptides, the growth-hormone-releasing hormone (GHRH) and somatostatin, which control the release of GH by the somatotropes of anterior pituitary. The GHRH, sleep, intense physical exercise, hypoglycemia and low levels of circulating IGF-1 stimulates the release of GH. However, this is inhibited by the somatostatin, hyperglycemia and increased blood concentrations of IGF-1. The GH acts on hepatocytes stimulating production and secretion of IGF-1, growth factor that stimulates the growth and differentiation of skeletal muscle tissue and also the overall growth of bone tissue [3;19-21].The availability of IGF-1 is regulated by binding proteins (IGF-BP); mostly is complexed to IGF-BP3. In skeletal muscle also occurs the production of IGF-1, which acts in an autocrine and paracrine pathways [22].

The main route of intracellular signaling of IGF-1 starts with its binding to the IGF1R, receptor formed by a dimer of two glycoproteins with cytoplasmic domains of tyrosine kinase. The interaction of IGF-1 and its receptor induces a tyrosine autophosphorylation in the cytoplasmic domains of the receptor, triggering an intracellular signaling cascade that promotes the survival and proliferation of the

muscle cell. Thus, IGF-1 has others effects, that go beyond the performance boost, may cause the development and progression of tumors [19].

In gene doping, multiple copies of the gene coding for the IGF-1 are inserted in skeletal muscle, promoting an increase in the muscle mass, due to hypertrophy of muscle cells. This somatic gene insertion can be accomplished through the use of two different vectors: plasmids or viruses. The plasmids are a more economical method, although less efficient. Since the viruses, widely used in gene therapy, insert the foreign DNA into the genome of the target cell. The viral classes commonly used are: (1) the adenovirus, with double-stranded DNA, and (2) the adeno-associated viruses, with single-stranded DNA [3;22].

3.4 Myostatin: a negative regulator of muscle growth and differentiation

First described by McPherron et al., the myostatin, member of the superfamily of transforming growth factor (TGF)-β is a potent negative regulator of growth and differentiation of skeletal muscle, where its gene expression predominates [23;24]. Just as the endocrine hormones, myostatin circulates in the blood plasma at high concentrations. Its inhibition, either through antibodies to myostatin, drugs what binds to it or the technology of knockout, results in a hypertrophy of skeletal muscle tissue, principle used in gene doping [25]. Many studies show these effects of increased performance provided by changes in the activity of myostatin. Among these, is the analysis performed by Mosher et al. in which was evaluated the relationship between mutations in the myostatin gene and increased muscle mass and racing performance in heterozygous dogs [26].

The muscular dystrophies are a heterogeneous group of congenital disorders characterized by severe muscle weakness, atrophy and destruction of muscle fibers. These diseases are submitted to three different therapies: genetic, cellular and pharmacological. In gene therapy, the defective genes are replaced by foreign genes, with the use of insertion vectors. The cell therapy is based on the replacement of defective muscle cell through the differentiation of pluripotent stem cells, including satellite cells found near the muscle fibers, cells of hematopoietic population and mesoangioblasts. So there are pharmacological treatments, which consist of the use of steroids, creatine and inhibitors of prostaglandin synthesis. The use of positive growth factors for myoblasts, like IGF-1, and the blockade of negative regulators of

muscle growth, such as myostatin, increases muscle mass and decrease the phenotypic manifestation of dystrophies. Several substances block the inhibitory activity of myostatin in muscle growth and differentiation. Among these, are emphasized the follistatin, its coding gene and some proteins that contain domains of follistatin, all with the ability to bind to myostatin, which makes it unavailable for its natural inhibitory function.

Still, other applications of myostatin are present in literature. High levels of myostatin are seen in skeletal muscles of elderly, indicating that this is a biological marker associated with age. With advancing age, there is a loss of muscle mass and strength in clinical cachexia, sarcopenia and muscle atrophy. Thus, the administration of myostatin antagonists has great value in treating these disorders. The inhibition of myostatin activity also promotes the fall of adipogenesis with aging, minimizing the effects of obesity and diabetes mellitus type 2 [27]. Besides the use of myostatin antagonists in therapy for muscular disorders and metabolic syndromes, they are also commonly used in gene doping for performance enhancement in sports competition. The inhibiting of the natural function of myostatin leads to hypertrophy of muscle cells, increasing the muscle mass.

4. The Detection of Gene Doping: Challenges and Limitations

The detection of gene doping is very difficult, although it can be accomplished by either direct or indirect methods. Direct methods involve the detection of recombinant proteins or gene insertion vectors - viruses or plasmids. Indirect methods consist in an analysis of the immune system responses to gene transfer and/or of changes in the transcriptome of a particular cell type.

A direct method to distinguish the purpose of gene modifications is the analysis of gene expression. When used for gene therapy, the transgene replaces a defective gene and, thus, gene expression is detected where it was previously lacked. However, in gene doping, the changes promote an increased concentration of gene product that already exists in a normal quantity, might induce different post-translational modifications [28]. A study performed by Lasne et al. showed some structural differences between endogenous and recombinant erythropoietin, responsible for their different isoelectric behavior [29]. Another possibility for direct detection is based on the use of molecular tests to differentiate the genomic DNA

from the cDNA. The sequence of complementary DNA does not contain introns, may be distinguished using the techniques of polymerase chain reaction (PCR) or Southern Blotting.

The detection of insertion vectors in blood plasma presents great difficulties, considering the extremely short half-life of circulating plasmids, adenoviruses and adeno-associated viruses. So, the only way to detect the insertion vectors in bodily fluids through the application of molecular tests with relatively short intervals, with the need to create a regular testing regime.

The use of indirect methods is an alternative strategy based on analysis of gene doping effects on cells, tissues or entire organism such the immune system responses to gene insertion vectors or 'non-self' peptides encoded by introduced nucleic acid [28].

Recently, the use of transcriptomics, which consists in analysis of tissue's complement of mRNA transcripts, became a promissory method to detect gene doping. The quantity and composition of a tissue's transcriptome is highly reflective of metabolic activity. Some tissues can be easily accessed to construct a pattern of gene expression. So, it's possible to estimate some changes in each tissue's pattern, enabling the development of doping detection techniques [30].

5. Conclusions

The scientific development tests human reason and ethics. Great advances, such as the advent of "omics sciences", provide new alternatives for the treatment of many diseases, but aggregate inappropriate opportunities that destroy and corrupt human values.

Science has elucidated genomes, mechanisms of gene regulation and metabolic pathways. These discoveries enabled the use of gene doping, a process that would hide the paths of fraud at the genomic level. Therefore, the biotechnology must find reliable methods of detection for these misapplications of knowledge. Last but not least, athletes and sports physicians must think about the real purpose of sports. There will always be a choice.

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2. ARTIGO II

Effect of rHuEPO and erythropoietin gene transfer on reproductive parameters and of rabbits

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Effect of rHuEpo and erythropoietin gene transfer in reproductive parameters of rabbits

T. F. Collares[1]; G. Urtiaga[1]; V. F. Campos[1]; P. M. M. Leon[1]; M. G. Amaral[2]; .F. K. Seixas[3]; T. Collares[1]; O. Dellagostin[4]; J. C. Deschamps[1]

[1] Laboratório de Embriologia Molecular e Transgênese, Centro de Desenvolvimento Tecnológico (CDTec), Universidade Federal de Pelotas, Pelotas, RS, Brazil

[2] Laboratório de Imunohistoquímica, Centro de Desenvolvimento Tecnológico (CDTec), Universidade Federal de Pelotas, Pelotas, RS, Brazil

[3] Laboratório de Genômica Funcional, Centro de Desenvolvimento Tecnológico (CDTec), Universidade Federal de Pelotas, Pelotas, RS, Brazil

[4] Laboratório de Biologia Molecular, Centro de Desenvolvimento Tecnológico (CDTec), Universidade Federal de Pelotas, Pelotas, RS, Brazil

***Corresponding Author**

Tiago Collares

Centro de Desenvolvimento Tecnológico

Universidade Federal de Pelotas

Campus Universitário s/nº, CEP 96010-900

Caixa Postal 354, Pelotas, RS, Brazil

Phone: +55 53 3275 7588, FAX: +55 3275 7551

E-mail: collares.t@gmail.com

Abstract

The aim of this study is to evaluate the effects of the administration of recombinant erythropoietin (rHuEpo) and erythropoietin gene transfer in reproductive parameters of rabbits. Fifteen rabbits were divided in 3 groups: group I (rHuEpo) received subcutaneously 25UI/kg of recombinant human erythropoietin, three times a week for 5 weeks; group II (pTarget/Epo) received a single dose of recombinant vector with the gene of the rabbit erythropoietin; group III (pTarget) received a single dose of empty pTarget vector (control). Throughout the experiment, reproductive and blood parameters were monitored, such as: sperm motility, spermatic vigor, sperm concentration, sperm viability, sperm morphology, erythrocytes and hematocrit level. Erythropoietin gene transfer and rHuEpo administration caused a significant increase in the number of erythrocytes. The animals which received rHuEpo showed an increase in the hematocrit level, reaching numbers between 41,34 and 52,32. The statistical analysis proved that the treatment and the time did not interfer on sperm motility, sperm concentration and spermatic vigor ($P<0.05$). The percentage of morphologically normal cells in group I as well as in group II decreased over time, however, there was no statistical difference between the treatments ($P<0.05$). This study is the first to show the answers to the use of gene doping with the erythropoietin gene and the rHuEpo administration in reproductive and blood parameters.

Key-words: gene doping; rHuEpo; gene therapy; reproductive parameters

Introduction

Erythropoietin (EPO) is an essential growth factor for the red cell lineage, controlling the survival, proliferation and differentiation of erythroid precursors. Inadequate erythropoietin production, as observed in patients with end-stage renal disease, results in an anemia (Lacombe and Mayeux, 1998). Genetic engineering has enabled the production of recombinant human erythropoietin (rHuEpo) to treat this and other diseases. Unfortunately, rHuEpo is misused by athletes to stimulate erythropoiesis, increasing, therefore, blood oxygen capacity and endurance capacity (Jelkmann, 2003).

In addition to this main focus of rHuEpo treatment, (Reichel and Gmeiner, 2010) described secondary benefits: increased exercise tolerance, normalization of increased cardiac output, improved quality of life, among others. In addition, some possible adverse effects were described, such as: hypertension, vascular access thrombosis and pure red cell aplasia (anti-EPO antibodies). In case of doping with rHuEpo, it is assumed that adequate medical supervision is not present during the treatment, posing, therefore, health risk for the athletes.

One of the modern techniques of molecular biology applied to health is gene therapy. Although, there are different definitions for the gene therapy, it is, essentially, the manipulation of the expression of specific genes, considering the nature of the disease. The strategies in gene therapy are designed to treat or prevent infectious or degenerative diseases, cancer, inherited or immune system disorders (Gatzidou et al, 2009).

As explained by Friedmann et al (2010) recently the successful development of gene therapy has provided the concepts, tools, opportunity, and, for some, the justification for genetic modification of functions that affect normal human aspects, including athletic performance. The potential misuse of this type of therapy is regarded as doping: gene doping.

Gene doping, thus, is based on the introduction and subsequent expression of a target gene into a host. It also involves the modulation of expression of endogenous genes. *In vivo* or *ex vivo* methods can be used for the introduction of the target gene into the athlete's body (Azzazy et al, 2009). The World anti-Doping Agency (WADA) prohibits this as “the non-therapeutic use of cells, genes, genetic

elements, or the modulation of gene expression, having the capacity to enhance athletic performance" (Wells, 2009).

Although the means of detecting this type of doping are being widely studied, there is still no efficient technique. The strategies used for detecting gene doping can be direct (evidence of doping agent) or indirect (evidence of consequences of *gene doping*) (McKanna and Toriello, 2010).

Many genes with potential to enhance athletic performance are available, such as erythropoietin, insulin-like growth factor, vascular endothelial growth factor, growth hormone and myostatin. These genes not only have the potential to improve athletic performance of human athletes, but they can also be applied in animal sports, such as horse racing (Haisma and Hon, 2006).

There are major current risks in gene therapy and potential gene doping, and three brief but serious examples are: cancers, severe autoimmune reaction to the product and reaction to the carrier virus (Sharp, 2010). Overexpression of erythropoietin has a number of potential safety risks. Administration of Epo causes an increase in hematocrit and this makes the blood more viscous and increases the load on the heart. Potential consequences include blockage of the microcirculation, stroke and heart failure (Wells, 2008).

The reflection of the use of EPO on reproductive parameters *in vivo* has not yet been described. On the other hand, in vitro studies with cultured cells have shown that erythropoietin stimulates steroidogenesis in Leydig cells, triggering an increase in testosterone production (Yamazaki et al, 2004).

Thus, the aim of this study was to evaluate the effects of erythropoietin gene transfer and rHuEpo administration on reproductive parameters of rabbits.

Materials and Methods

Animals and experimental design

Adult male New Zealand White rabbits weighing 4.5 to 5 kg used in all experiments were obtained from the Central Animal Facility of the Federal University of Pelotas (UFPel) and kept under conventional housing conditions. The animals were housed with appropriate bedding and provided free access to drinking water and food. Rabbits were kept in standard single cages under controlled temperature

and light conditions. The study protocol was approved and maintained in accordance with the guidelines of the Ethics Committee in Animal Experimentation of the UFPel.

Fifteen rabbits were randomly divided in 3 different groups: group I (rHuEpo) received subcutaneously 25UI/kg of recombinant human erythropoietin dissolved in normal saline three times a week for 5 weeks; group II (pTarget/Epo) received a single dose of recombinant vector with the gene of erythropoietin of rabbit; group III (pTarget) received a single dose of vector pTarget empty (control). Intramuscular DNA injection was performed essentially as previously described, but with modifications (Maruyama et al, 2001). A total of 400 µg of plasmid DNA was injected into the lateral sides of each lower leg (200 µg per each site). The recombinant human erythropoietin used in experiment was the Eprex (Issy-les-Moulinaux, France). All the groups, treatments and doses are described in Table 1.

Cloning of rabbit EPO cDNA

Two rabbits were euthanatized with sodium pentobarbital (200mg/kg), which was injected intravenous. Subsequently, whole kidneys were resected and immediately frozen in liquid nitrogen until their use. The total RNA was isolated with TRIzol® Reagent (Invitrogen™, Carlsbad, USA), according to the recommended protocol. The final RNA concentrations were determined by the QuBit® fluorometer (Invitrogen™, Carlsbad, USA) and 2µg was used to the synthesis of first strand cDNA performed with SuperScript™ III Reverse Transcriptase (Invitrogen™, Carlsbad, USA) according to the manufacturer's protocol. Primers sets were designed with the Primer Express v. 3.0 software (Applied Biosystems™, USA) to amplify EPO cDNA sequences of rabbit (GenBank accession no. AF290943.1) based on conserved EPO DNA sequence among several mammals, as follows: FOR - 5' ATGGGGGCGCGCGGACGC 3' and REV - 5' TCACCTGTCCCCTCTCCTGCAGGC 3'. The PCR parameters were 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, with an additional initial 15 min denaturation at 95°C and a 10 min final extension at 72°C. PCR amplification from rabbit kidney cDNA produced a DNA fragment of 588bp. PCR products were sequenced using a MegaBACE 1000 automatic sequencer (Amersham Biosciences, USA).

Plasmid vectors

The PCR products were purified with illustra GFX™ PCR DNA and Gel Band Purification Kit (Amershan®) and then were cloned into plasmid pTARGET contained in pTARGET™ Mamalian Expression Vector System (Promega®, USA), according to the manufacturer's instructions. After transformation into *E. coli* TOP10, colonies grown in competent cells were picked and recombinant DNA plasmids were isolated using the Plasmid Miniprep procedure (Sigma, USA). The colonies were screened for correct insert direction and length with bacteria colony PCR and digestion with EcoRI. The selected clone was sequenced by automatic sequencer. This clone was cultivated and submitted to the DNA extraction on a large scale using the Perfectprep Plasmid Maxi Kit (Eppendorf®, Germany).

Semen collection and evaluations

One ejaculate per male was collected each week using an artificial vagina. The volume of fresh semen was measured in a graduated conical tube. The percentage of motile sperm was evaluated subjectively from samples diluted 1:10 in Ringer with sodium lactate (BASA Pharmaceutical Industry, BRA). The motility percentage and spermatic vigor was estimated at 37°C and observed using a microscope with phase-contrast optics at a magnification of 200x. The microscope was connected to a video camera and the motility samples were examined by two experienced technician. The concentration sperm was measured by a Neubauer counting cell chamber. The sperm viability was assessed by using a Live/Dead Sperm Viability kit (Molecular Probes, Inc.) according to the manufacturer's instructions. The visualization was performed with a 200x objective on a fluorescence microscope (Olympus BX 51). One hundred spermatozoa from each sample were counted to determine the percentage of negative and positive spermatozoa. In order to evaluate the morphology of each sample, it was prepared smears and they were stained with hematoxylin-eosin. Subsequently, the sperm was analyzed under a light microscope with 200x objective, and it was observed the sperm defects, considering the count of 100 cells. Individual spermatozoa were classified as having normal or abnormal morphology (including head, neck, and tail defects).

Blood Parameters

Blood samples were collected on the first day of the experiment, before the injections, once every week and at the end of the experiment. Blood samples (1.0 ml) were obtained from the auricular artery. The blood count test was performed by Ary Costa Laboratory (BRA), using the automated method ABX Micros 60 and microscopy.

Histological analysis

The animals were sacrificed at the end of experiment through injectable chemical method using intravenous Pentobartital in the dose of 200mg/kg. The testis were dissected, fixed in 10% buffered formaldehyde for 24 hours, embedded in paraffin and processed for analysis under light microscopy (LM). Later, they were stained with hematoxylin-eosin for the detection of possible tissue injury.

Statistics

All calculations were performed using SAS statistical software (SAS Institute Inc., Cary, NC). The experimental design used was ANOVA with split plots in time. Analysis of variance with Tukey correction was performed for the test of average comparison. Furthermore, we calculated the Pearson correlation coefficient among the following variables. Statistical significance was set at $P<0.05$.

Results

Blood Analysis

Injection of pTarget/Epo significantly increased the levels of erythrocytes on week 1 ($P<0.05$). On week 2, the hematocrit levels of pTarget/Epo rabbits lowered and on week 3 returned to the pre-injection level (Figure. 1A – Table 4). In the rHuEpo group, the number of erythrocytes decreased on week 1 and then increased over time. Consequently, it is possible to observe that the rHuEpo administration caused a significant increase in the number of erythrocytes. Group III, the control

group, maintained stable levels of red blood cells. This way, differences were observed regarding time and treatments ($P<0.05$). Additionally, the hematocrit level on week 1 was significantly higher in the pTarget/Epo group than in others groups and on week 4 the rHuEpo group had the highest hematocrit level (Figure 1B – Table 5). Statistical difference was observed between the control and treatment groups.

Reproductive parameters

The statistical analysis showed that both treatments as well as time had no effect ($P<0.05$) on sperm motility, sperm concentration and spermatic vigor. The semen viability of rHuEpo rabbits decreased over time, with significant differences starting on week 3 ($P<0.05$) (Table 2). The percentage of cells with normal morphology of both rHuEpo and pTarget/Epo groups started decreasing on week 2 and showed significant difference on week 4. There was no statistical difference between treatments ($P<0.05$). (Figure 2 – Table 6). Moreover, in the morphological evaluations it was observed in rHuEpo and pTarget/epo rabbits that the middle piece defects increased over time. However, there was a change in head defects only in rHuEpo rabbits, observing a reduction of this abnormality over time. It was not observed any alteration regarding defects of the tail. The Pearson correlation coefficients between the analyzed parameters are presented in Table 3.

Histological analysis

No histological alteration was found in the analysis of the testis of rabbits. It is possible that due to the time that the samples were collected, no pathologies were observed, as the tissue probably had already regenerated.

Discussion

One of the most serious problems of current competitive sport is the increasing abuse of various performance-enhancing substances. Advances in recombinant DNA technology have created one of the most powerful weapons in the doping arsenal: recombinant proteins (Sweeney, 2004; Unal and Ozer, 2004). Thus, with the advances in molecular biology techniques, attention was drawn to gene therapy and

its misuse in sports. There are some risks in gene doping, as not only the product and the procedures for delivery of the product carry risks, but also the uncontrolled expression of the genes may themselves be harmful (Wells, 2008).

In our study, we analyzed the different reproductive and blood parameters to assess the degree of impairment of reproductive potential from biological models subjected to gene doping with erythropoietin and to administration of recombinant erythropoietin.

In blood tests we observed significant differences in levels of hematocrit and erythrocytes in both treatments over time (pTarget/Epo; rHuEpo), and no alterations in the control group. Additionally, a correlation was detected between these parameters. However, analyzing the averages among the groups, it was observed that the averages showed significant differences between treatments and control.

A significant increase in hematocrit was observed in mice injected with more than 100 µg of plasmid (Rizzuto et al, 1999). In other previous findings hematocrit did not change in rats that were simply injected with 400 µg of pCAGGS-Epo without *in vivo* electroporation (Maruyama et al, 2000). In our study, the levels of hematocrit and erythrocyte in pTarget/Epo, group showed a significant increase on week 1 and later a decrease to pre-injection levels. This suggests that there was no long-term expression of Epo probably due to electroporation.

Previous studies have evaluated the influence of the use of rHuEpo in reproductive parameters demonstrating that the recombinant erythropoietin stimulates the production of testosterone in vitro (Yamazaki et al, 2004), this being consistent with other findings. The effects of Epo on testicular functions have been investigated. The kidney is known to be the major site of EPO production in the body, however in a mouse model EPO synthesis occurred at the epididymis (Kobayashi et al, 2002).

Patients with chronic renal failure developed anemia secondary due to inefficient Epo synthesis with accompanying poor testicular functions, which are improved by Epo treatment (Dobashi et al, 2005; Lawrence et al, 1997; Yamamoto et al, 1997), but studies are necessary in healthy subjects.

Foresta et al (1994) reported that intravenous injection of rHuEpo improved testicular testosterone production. These findings suggest that Epo may directly influence human Leydig cell function. However, the increase in testosterone synthesis, may lead to a negative feedback regarding the release of GnRH/LH and,

therefore, affect directly and indirectly reproductive parameters and sperm, potentially triggering a temporary or permanent infertility.

In reproductive parameters of motility, sperm concentration and spermatic vigor, the analysis in our study did not show significant differences after the animals were under treatment. In the rHuEpo group, the viability of sperm decreased over time, but there was no significant difference in relation to the control group. It was also observed the correlation between sperm motility, concentration, vigor, morphology and viability.

In the analysis of the morphology, despite the statistical differences in the groups treated, there was no difference in the control group, which may indicate an effect of the environment. According to previous reports (Kuzminsky et al, 1996) on the abnormalities of rabbit spermatozoa, our findings are consistent with an acceptable number of abnormal cells. In our study, there was no relation between blood and reproductive parameters, only a weak correlation between levels of hematocrit and erythrocyte to the number of abnormalities in the head.

Conclusion

The present study is the first to report the evaluation of reproductive and blood responses to the use of gene doping and administration of rHuEpo. Gene transfer of Epo significantly increased levels of red blood cells and decreased number of cells with normal morphology. The administration of rHuEpo caused a significant increase in number of erythrocytes and hematocrit levels and a decrease in the viability and number of sperm cells with normal morphology. Treatments and time did not affect the motility, concentration and spermatic vigor.

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Table 1. Scheme of inoculations, dosages and routes of administration in different groups of the experiment.

Groups	Animals	Dose	Route of administration
rHuEpo	5	75 UI/Kg/week	subcutaneous
pTarget/Epo	5	1x400 µg	intramuscular
pTarget (Control)	5	1x400 µg	intramuscular

Table 2: Average of sperm viability (%) rabbits subjected to different treatments in terms of time

Groups	0	1	2	3	4	5
pTarget/EPO	92.20^{Aab}	89.60^{Aa}	89.40^{Aa}	79.60^{Aa}	82.00^{Aa}	80.00^{Aa}
rHuEPO	93.00^{Aa}	91.50^{Aa}	83.40^{ABa}	72.60^{ABa}	74.40^{ABa}	69.20^{Ba}
Control	74.75^{Ab}	69.50^{Ab}	73.25^{Aa}	72.25^{Aa}	81.67^{Aa}	69.00^{Aa}

Means followed by same capital letters horizontally are not statistically different from each other.

Means followed by same lowercase letters vertically were not statistically different from each other

$P<0.05$

Table 3. Pearson correlation coefficients among tested variables

	MOT	VIGOR	CONC	VIAB	NORM	HEAD	P.I.	TAIL	ERY
VIGOR	0,68**	-	-	-	-	-	-	-	-
CONC	0,25*	0,03**	-	-	-	-	-	-	-
VIAB	0,48**	0,40**	0,23*	-	-	-	-	-	-
NORM	0,21*	0,27*	0,12	0,57**	-	-	-	-	-
HEAD	-0,09	-0,10	-0,19	-0,32**	-0,39**	-	-	-	-
P.I.	0,00	-0,03	-0,16	-0,02	0,04	0,72**	-	-	-
TAIL	-0,12	-0,23*	0,03	-0,34**	-0,86**	0,30**	-0,08	-	-
ERYT	0,10	0,04	-0,14	-0,13	-0,20	-0,16	-0,21*	0,05	-
HEM	0,19	0,10	-0,05	-0,09	-0,21*	-0,18	-0,24*	0,13	0,92**

MOT: sperm motility; VIGOR: spermatic vigor; CONC: sperm concentration, VIAB: viable sperm; NORM: normal sperm; HEAD: abnormalities in the head; PI: abnormalities in the middle piece; TAIL: abnormalities in the tail; ERYT: number of erythrocytes; HEM: hematocrit level - ** P<0,01; * P<0,05

Figure 1. Time-course of blood parameters after pTarget/Epo transfer and administration of rHuEpo in rabbits. A) Number of erythrocytes B) Hematocrit levels

A)

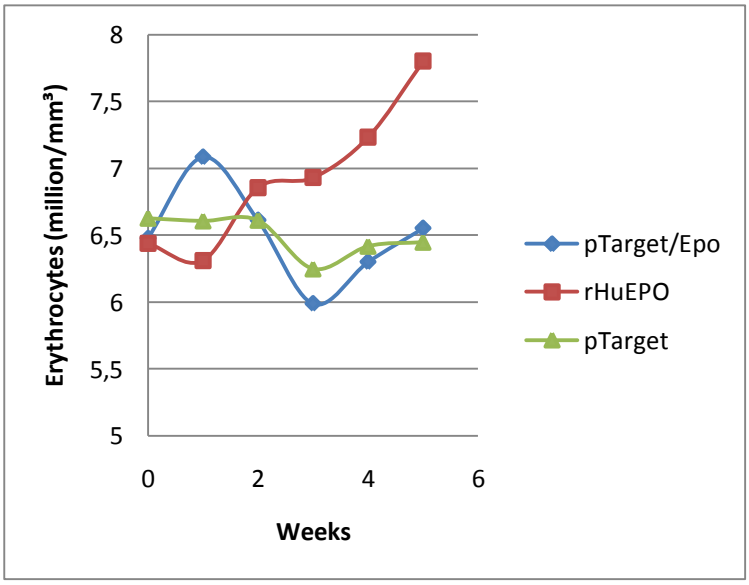

Table 4: Average number of erythrocytes (millions/mm^3) rabbits subjected to different treatments in terms of time.

Groups	1	2	3	4	5	6
pTarget/EPO	6,48^{BCa}	7,08^{Aa}	6,61^{ABa}	5,99^{Cb}	6,30^{BCb}	6,55^{Bb}
rHuEPO	6,44^{CDa}	6,31^{Db}	6,86^{BCa}	6,93^{BCa}	7,23^{Ba}	7,80^{Aa}
Control	6,63^{Aa}	6,61^{Ab}	6,61^{Aa}	6,25^{Ab}	6,42^{Ab}	6,45^{Ab}

Means followed by same capital letters horizontally are not statistically different from each other.
Means followed by same lowercase letters vertically were not statistically different from each other.
P<0.05

B)

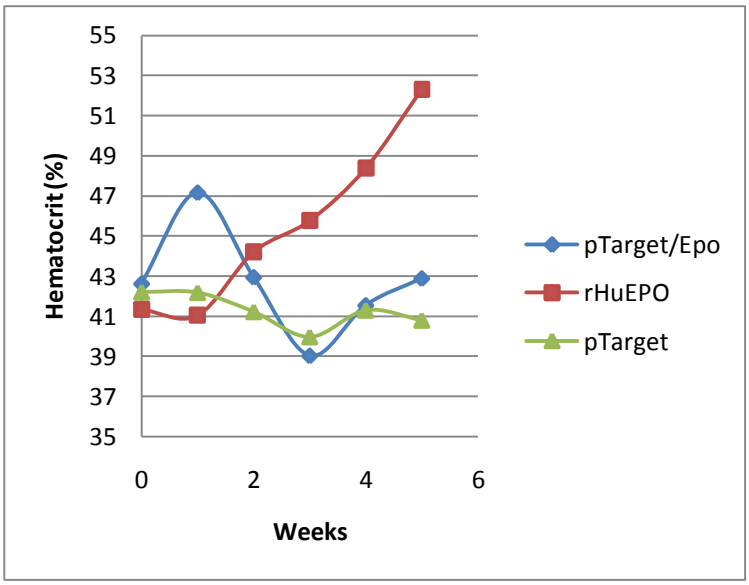

Table 5: Average of hematocrit levels (%) rabbits subjected to different treatments in terms of time.

Groups	1	2	3	4	5	6
pTarget/EPO	42,62^{Ba}	47,16^{Aa}	42,94^{Bab}	39,02^{Cb}	41,54^{BCb}	42,88^{Bb}
rHuEPO	41,34^{Da}	41,04^{Db}	44,20^{CDa}	45,76^{BCa}	48,38^{Ba}	52,32^{Aa}
Control	42,20^{Aa}	42,18^{Ab}	41,23^{Ab}	39,98^{Ab}	41,30^{Ab}	40,80^{Ab}

Means followed by same capital letters horizontally are not statistically different from each other.
Means followed by same lowercase letters vertically were not statistically different from each other.
P<0.05

Figure 2. Time-course of sperm cells with normal morphology after pTarget/Epo transfer and administration of rHuEpo in rabbits.

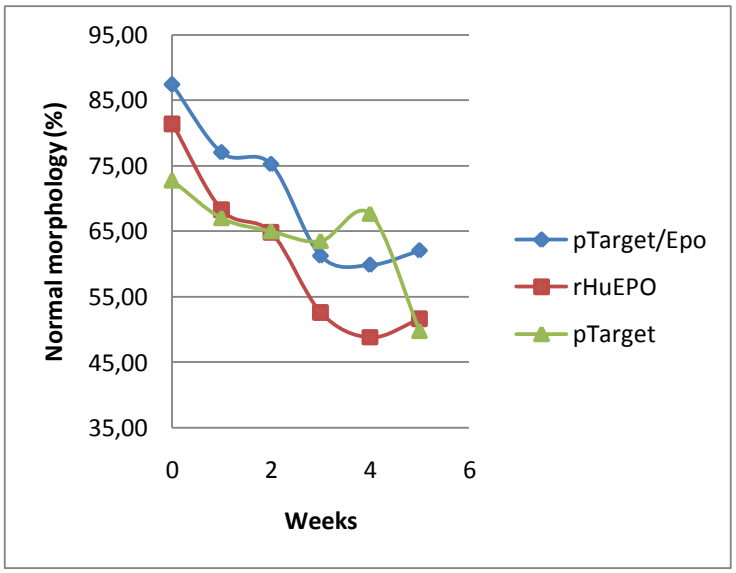

Table 6: Average of normal sperm cell rabbits (%) subjected to different treatments in terms of time.

Groups	0	1	2	3	4	5
pTarget/EPO	87.40^{Aa}	77.00^{ABa}	75.20^{ABa}	61.20^{Ba}	59.80^{Ba}	62.00^{Ba}
rHuEPO	81.40^{Aa}	68.25^{ABa}	64.80^{ABa}	52.60^{Ba}	48.80^{Ba}	51.60^{Ba}
Control	72.75^{Aa}	67.00^{Aa}	65.00^{Aa}	63.50^{Aa}	67.67^{Aa}	49.75^{Aa}

Means followed by same capital letters horizontally are not statistically different from each other.
Means followed by same lowercase letters vertically were not statistically different from each other.
P<0.05

Figure 3. Time-course of number of sperm cells with abnormal morphology in middle piece after pTarget/Epo transfer and administration of rHuEpo in rabbits.

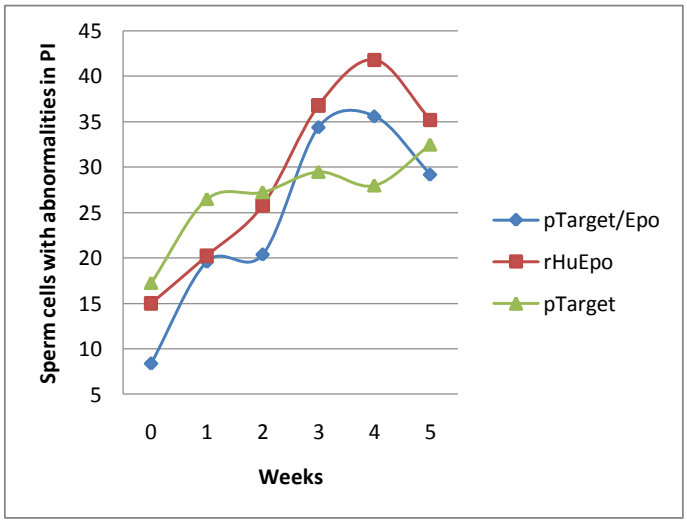

Table 7: Average of abnormalities in the middle piece of sperm cells rabbits subjected to different treatments in terms of time.

Groups	0	1	2	3	4	5
pTarget/EPO	8.40^{Ba}	19.60^{ABa}	20.40^{ABa}	34.40^{Aa}	35.60^{Aa}	29.20^{ABa}
rHuEPO	15.00^{Ba}	20.25^{ABa}	25.80^{ABa}	36.80^{ABa}	41.80^{Aa}	35.20^{ABa}
Control	17.25^{Aa}	26.50^{Aa}	27.25^{Aa}	29.50^{Aa}	28.00^{Aa}	32.50^{Aa}

Means followed by same capital letters horizontally are not statistically different from each other.
Means followed by same lowercase letters vertically were not statistically different from each other.
P<0.05

3. CONCLUSÃO

Este é o primeiro estudo que avaliou as respostas reprodutivas e sanguíneas com o uso do doping genético e administração de rHuEpo. A transferência gênica da Epo aumentou significativamente os níveis de eritrócitos e diminuiu o número de células com morfologia normal. A administração da rHuEpo causou um aumento significativo no número de eritrócitos e do hematócrito e uma diminuição na viabilidade e no número de células espermáticas com morfologia normal. Os tratamentos e o tempo não interferiram na motilidade, concentração e vigor.

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