HEMORHEOLOGICAL AND CARDIOVASCULAR EFFECTS OF ERYTHROPOIETIN IN A RAT MODEL OF SPORTS DOPING / EFEITOS HEMORREOLÓGICOS E CARDIOVASCULARES DA ERITROPOIETINA NUM MODELO DE RATO EM EXERCÍCIO FÍSICO SOB A ACÇÃO DE DROGAS

Piloto N[1], *Teixeira HM*[2], *Garrido P*[1], *Teixeira-Lemos E*[1], *Teixeira M*[1], *Parada B*[1,3], *Sereno J*[1], *Pinto R*[4], *Alves R*[5], *Santos P*[6], *Romão AM*[1], *Nunes S*[1], *Neto P*[7], *Carvalho L*[7,8], *Couceiro P*[8], *Xavier F*[7], *Rocha-Pereira P*[9], *Costa E*[10,11], *Belo L*[11,12], *Santos-Silva A*[11,12], *Teixeira F*[1,11], *Reis F*[1,11]

ABSTRACT

Recombinant human erythropoietin (rhEPO) has been therapeutically used for correction of anaemia. However, due to the increase in circulating red blood cells (RBCs) it promotes, thus increasing oxygen delivery to muscles and improving performance in sport, it has been also illegally used as sports doping. Besides the well known increase of hematocrit and blood viscosity, which might cause serious complications for the athletes, other disturbances could occur, whose mechanisms remain to be fully elucidated. This study aimed to evaluate the hemorheological and cardiovascular effects of administration of rhEPO to rats under chronic aerobic exercise. A ten week-protocol was performed in four male Wistar rat groups: control – sedentary; rhEPO – 50 IU/kg, 3 times/wk; exercised (EX) – swimming for 1hr, 3 times/wk; EX+rhEPO. rhEPO in trained rats promoted erythrocyte count increase, hypertension, heart hypertro-

[1] Institute of Pharmacology & Experimental Therapeutics, IBILI, Medicine Faculty, Coimbra University
[2] Forensic Toxicology Laboratory, North Branch of the National Institute of Legal Medicine, Porto
[3] Service of Urology and Renal Transplantation, Coimbra University Hospital
[4] Pharmacology and Pharmacotoxicology Unit, Pharmacy Faculty, Lisbon University
[5] Service of Nephrology, Coimbra University Hospital
[6] Functional Genomics Laboratory, Center of Histocompatibility of the Centre, Coimbra
[7] Service of Anatomic Pathology, Coimbra University Hospital
[8] Institute of Anatomic Pathology, Medicine Faculty, Coimbra University
[9] Research Centre for Health Sciences, Beira Interior University, Covilhã
[10] Institute of Health Sciences of University Catholic, Porto
[11] Institute for Molecular and Cellular Biology, Porto University
[12] Biochemistry Department, Pharmacy Faculty, Porto University, Portugal

Corresponding author:
Flávio Reis, PhD
Institute of Pharmacology and Experimental Therapeutics, IBILI,
Medicine Faculty, Sub-Unit 1 (Polo III), Coimbra University, 3000-354 Coimbra, Portugal
Tel: +351 239480053; Fax: +351 239480065;
E-mail: freis@fmed.uc.pt

phy, sympathetic and serotonergic overactivation, as well as a trend to increased oxidative stress. In conclusion, rhEPO doping in rats under chronic exercise promotes not only the expected increased hematocrit, but also other serious deleterious cardiovascular and thromboembolic modifications, including live risk, which might be known and assumed by all sports authorities, including athletes and their physicians.

(Key-words: *rhEPO, doping, chronic aerobic exercise, hemorheological and cardiovascular effects).*

INTRODUCTION

Recombinant human erythropoietin (rhEPO) doping remains a huge concern, not only because of distortion of sport truth, but particularly because of the risks for athletes under doping administration. Besides the well known increase of hematocrit and blood viscosity, other hemorheological and cardiovascular disturbances could occur, but the mechanisms underlying remain to be elucidated.

Erythropoietin (EPO) is a glycoprotein hormone synthesized predominantly in the kidneys which stimulates proliferation and maturation of erythroid cells in the bone marrow[1]. The production of recombinant human erythropoietin (rhEPO), which has been widely used for correction of anaemia, allowed many patients for the first time to resume their normal daily activities due to increased energy[2]. The increase in circulating red blood cells (RBCs) may be used to increase oxygen delivery to muscles, improving performance in sport[3]. The availability of rhEPO allowed its use in doping. As soon as the anti-doping authorities were able to distinguish between the endogenous and the rhEPO[4], the scandal of its use in sport was revealed, with particular emphasis to cycling and cross-country skiing, between other sport modalities[5-6]. Sports authorities prohibited the use of rhEPO in 1988. The idea was, first, to limit the degree of health risk and, second, the degree of performance enhancement.

Athletes who abuse rhEPO consider only the benefit to performance and usually ignore the potential short and long-term liabilities[7-8]. In the early 1990s, there was a considerable speculation about the involvement of rhEPO doping in the death of professional cyclists[8-9]. The artificial increase in RBC count and haematocrit, further enhanced by dehydratation during prolonged exercise, predisposes to thromboembolic complications, which might be connected to sudden death in sport practice[10]. However, the cellular/molecular mechanisms underlying those sudden death episodes are poorly clarified, as well as whether rhEPO use was linked to this outrageous phenomenon.

The purpose of this study was to evaluate the hemorheological and cardiovascular effects of rhEPO treatment on rats under chronic aerobic exercise.

MATERIAL AND METHODS

Animals and experimental protocol

Male Wistar rats (Charles River Laboratories Inc., Barcelona, Spain),

weighting 220-250g, were maintained in an air conditioned room (22-24ºC) with humidity of 60%, subjected to 12-h dark-light cycles and given standard rat chow (AO4, Panlab, Letica, Barcelona, Spain) and water *ad libitum*. All experiments with animals were performed in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Council of Europe n.er 123, Strasbourg, 1985), as well as with ethical laws of the National Institutions for Science and Technology.

After a period of adaptation of at least 2 weeks, four groups (n=8 each) were evaluated for 10 weeks-treatment: a) control – sedentary; b) rhEPO – 50 IU/Kg/3x/wk of s.c. beta-EPO (Recormon®, Roche Pharmaceuticals); c) exercised (EX) – swimming training (1 hr, 3x/wk); d) swimming exercised + rhEPO (EX+rhEPO). The swimming groups were submitted to a previous adaptation period of 1 week in order to minimize the stress caused by water. These sessions started with 15 min in the first day, with an increment of 5 min each day until achieved a period of continuous 60 min. After this period, the exercise was performed for 1 h, 3x/week, in a temperature-controlled bath set at 35±1ºC, for 10 weeks.

Body weight (BW) was monitored during the study, and blood pressure (BP) and heart rate (HR) measured using a tail-cuff sphygmomanometer LE 5001 (Letica, Barcelona, Spain).

Sample collection and preparation

Blood: At the end of treatments the rats were subjected to intraperitoneal anesthesia with a 2 mg/kg BW of a 2:1 (v:v) 50 mg/mL ketamine (Ketalar®, Parke-Davis, Lab. Pfeizer Lda, Seixal, Portugal) solution in 2.5% chlorpromazine (Largactil®, Rhône-Poulenc Rorer, Lab. Vitória, Amadora, Portugal) and blood samples were immediately collected by venipuncture from the jugular vein into syringes without anticoagulant (for serum samples collection) or with the appropriate anticoagulant: EDTA, heparin or a solution of ACD (acid citrate-dextrose). Blood was centrifuged (160 *g* for 10 min. at 20ºC) to obtain platelet rich plasma (PRP), which was then centrifuged (730 *g* for 10 min. at 20ºC) to obtain the platelet pellet and poor platelet plasma (PPP).

Tissues: The rats were sacrificed by cervical dislocation and the heart, the adrenals, the kidneys, the liver and the gastrocnenius muscle were immediately removed, placed in ice-cold Krebs' buffer and carefully cleaned of adherent fat and connective tissue. The BW and the weights of heart (HW), left ventricle (LVW), adrenals (AW), kidney (KW), liver (LW) and muscle (MW) were measured in all the rats under study in order to be used as trophy indexes. The following tissues were removed from the rat that suffered a sudden death episode during an exercise session after 8 weeks of treatment: lungs, kidneys, brain, heart/left ventricle and liver. Tissues were analyzed for histomorphology with haematoxilin-eosin (H&E) staining.

Serum Epo concentration and haematological data

Serum erythropoietin was measured by using an immunoassay kit

(R&D Systems, Minneapolis, USA). Results were expressed in pg/ml. Red blood cell (RBC) count, haematocrit (Hct), haemoglobin (Hb) concentration, haematological indices [mean cell Hb (MCH) and mean cell Hb concentration (MCHC), mean cell volume (MCV) and red cell distribution width (RDW)], platelet count and platelets indices [plaquetocrit (PCT), mean platelet volume (MPV) and platelet distribution width (PDW)] were assessed in an automatic Coulter Counter® (Beckman Coulter Inc., USA, CA).

Renal and liver function and lipid profile

Serum creatinine, ureia and uric acid concentrations were used as renal function indexes and aspartate (AST) and alanine aminotransferase (ALT) levels were assessed for liver evaluation, through automatic validated methods and equipments (Hitachi 717 analyser).

Serum total cholesterol (Total-c) and triglycerides (TGs) were analysed on a Hitachi 717 analyser (Roche Diagnostics Inc., MA, USA) using standard methods.

Catecholamine and serotonin assay

Noradrenaline (NA) and adrenaline (A) concentrations in plasma, platelet, adrenals and brain, as well as plasma, platelet and brain 5-hydroxy-tryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) contents, were evaluated by high performance liquid chromatography with electrochemical detection (HPLC-ED), according to previously described[11], using appropriate standards (Sigma Chemical Co., St. Louis, MO, U.S.A.) and software (Gilson 710). Concentrations were expressed in: ng/ml for plasma and platelets and µg/g wet tissue for adrenals and brain.

Serum inflammatory profile and redox status

Inflammatory markers: Serum levels of interleukin 2 (IL-2), IL-1β, transforming growth factor β1 (TGF-β1) and tumour necrosis factor α (TNF-α) were measured by ultrasensitive Quantikine® ELISA kits (R&D Systems, Minneapolis, USA) and C-reactive protein (CRP) by using an ELISA kit from Helica Biosystems, Inc. (Fullerton, CA, USA). All assays were performed in duplicate.

Redox status: The thiobarbituric acid reactive-species (TBARs) assay was used to assess serum and muscle products of lipid peroxidation, via malondialdehyde (MDA), according to previously described[12]. Samples were analysed spectrophotometrically at 532 nm using 1,1,3,3-tetramethoxypropane as external standard. The serum concentration of lipid peroxides (in MDA) was expressed as µmol/l. Serum 3-nitrotyrosine (3-NT), which is an index of peroxynitrite formation, was measured through an enzymatic immunoassay (HyCult biotechnology b.v., Uden, Netherlands). Ferric reducing antioxidant potential (FRAP) assay was used to estimate serum total antioxidant status (TAS)[13].

Data analysis

For statistical analysis, we used the Statview 4.53 software from Abacus Concepts Inc. (Berkeley, CA, USA). Results are presented as mean ± standard error of the mean (s.e.m.). Comparisons between groups were performed using Factorial ANOVA and Fisher's test. Significance was accepted at *P* less than 0.05.

RESULTS

Serum EPO concentration and haematological data

Serum EPO concentrations at the end of treatments were significantly higher ($P<0.05$) in the rhEPO rats when compared with the controls, while no significant differences were encountered in the EX animals. rhEPO treatment in the exercised rats (group EX+rhEPO) did not significantly modify the serum EPO concentrations (Table I).

The rats under rhEPO treatment showed an increase in RBC count ($P<0.05$) when compared with the control. This was accompanied by increased ($P<0.05$) MCHC and MCV, together with a decreased MPV (Table I). The exercised rats demonstrated no relevant changes concerning haematological data, excepting higher MCHC and lower MPV vs the control animals. In the exercised rats treated with rhEPO, there was a statistically significant increment in RBC count, together with a trend to increased Hb and Hct vs the EX rats without rhEPO therapy. Platelet count and PCT also showed a trend to higher values in this group (Table I).

Table I – Serum EPO concentration and hemorheological data

Parameters	Sedentary		Swimming	
	Control (n=7)	rhEPO (n=7)	EX (n=7)	EX+rhEPO (n=7)
Serum [EPO] (pg/ml)	22.25 ± 1.00	35.75 ± 10.1[a]	27.83 ± 1.50	25.75 ± 2.24
Hemorheological data				
RBC count ($\times10^{12}$/L)	7.31 ± 0.16	7.67 ± 0.08[a]	7.59 ± 0.15	8.23 ± 0.14[b]
Hb (g/dL)	14.45 ± 0.65	14.11 ± 0.15	14.86 ± 0.25	15.45 ± 0.45
Hct (%)	41.45 ± 1.65	39.59 ± 0.45	41.40 ± 0.82	44.05 ± 1.45
MCH (pg)	19.80 ± 1.30	18.76 ± 0.22	19.60 ± 0.34	18.75 ± 0.25
MCHC (g/dl)	34.80 ± 0.20	35.75 ± 0.18[a]	35.93 ± 0.21[a]	35.05 ± 0.05
MCV (fl)	56.80 ± 3.50	52.48 ± 0.60[a]	54.61 ± 1.13	53.55 ± 0.85
RDW (%)	15.25 ± 0.65	15.89 ± 0.38	14.84 ± 0.55	14.25 ± 0.15
Platelet count ($\times10^{9}$/L)	904.0 ± 9.0	986.3 ± 41.5	1008.4 ± 35.9	1021.0 ± 57.0
PCT (%)	0.55 ± 0.02	0.56 ± 0.02	0.57 ± 0.02	0.60 ± 0.07
MPV (fL)	6.15 ± 0.25	5.69 ± 0.06[a]	5.63 ± 0.14[a]	5.85 ± 0.35
PDW (%)	16.85 ± 0.15	16.73 ± 0.18	16.37 ± 0.26	17.00 ± 0.10

Results are means ± s.e.m. of *n* rats per group. [a] $P<0.05$, [aa] $P<0.01$ and [aaa] $P<0.001$ vs the sedentary group (control); [b] $P<0.05$, [bb] $P<0.01$ and [bbb] $P<0.001$ *vs* the Swimming group without rhEPO (exercise: EX). EPO, erythropoietin; Hb, haemoglobin; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; MCV, mean corpuscular volume; MPV, mean platelet volume; PCT, plateletocrit; PDW, platelet distribution width; RBC, red blood cell; RDW, red deviation weight; rhEPO, recombinant human erythropoietin

Renal and liver function and lipid profile

Urea content was lower ($P<0.05$) in the rhEPO group vs control, without significant changes on creatinine and uric acid. Exercised rats also presented significantly lower values of urea ($P<0.05$) and uric acid ($P<0.01$). This reduction was prevented in the rats under exercise and rhEPO treatment (EX+rhEPO) (Table II).

Serum AST was unchanged between groups, and ALT was identical for all the groups, excepting for a lower value in the control animals (Table II).

Concerning the lipid profile, while the rhEPO rats presented a trend to higher Total-c contents and significantly increased TGs levels ($P<0.05$), the EX animals showed the opposite profile. The values encountered for the EX+rhEPO rats were similar to those of the EX animals (Table II).

Blood pressure and tissue weights

Blood pressure (SBP, DBP and MBP) and HR values were higher in the rfEPO group when compared with control. The same pattern was found for EX group. In exercised animals, rhEPO treatment further increased blood pressures ($P<0.001$) and HR ($P<0.05$) (Table III). Body weight showed a lower value in EX rats vs control, without further changes between groups. HW and HW/BW were significantly higher in rhEPO group vs control, together with significant lower LVW and LVW/BW. In the rats under exercise practise, rhEPO treatment promoted a further increment in HW and HW/BW, with a trend to increased values of LVW and LVH/BW (Table III). Concerning the other tissues, in the rhEPO group there was a significant reduction of ($P<0.05$) KW/BW, while in the rats under exercise there were higher values of LVW/BW, AW/BW and MW/BW (Table III).

Table II – Effects of rhEPO on renal function, liver function and lipid profile

Parameters	Sedentary		Swimming	
	Control (n=7)	rhEPO (n=7)	EX (n=7)	EX+rhEPO (n=7)
Renal Function				
Urea (mg/dL)	18.84 ± 0.55	17.37 ± 0.46^{a}	17.35 ± 0.26^{a}	18.60 ± 0.63
Creatinine (mg/dL)	0.57 ± 0.01	0.54 ± 0.02	0.57 ± 0.01	0.56 ± 0.01
Uric acid (mg/dL)	0.68 ± 0.05	0.77 ± 0.05	0.40 ± 0.06^{aa}	0.50 ± 0.03
Liver Function				
AST (IU/L)	27.20 ± 0.37	27.50 ± 0.50	30.60 ± 2.84	32.40 ± 2.27
ALT (IU/L)	50.20 ± 0.86	70.00 ± 2.28^{aaa}	65.20 ± 1.59^{aaa}	63.50 ± 2.63
Lipid Profile				
Total-c (mg/dL)	53.17 ± 1.66	55.00 ± 1.94	43.67 ± 1.20^{aa}	38.25 ± 1.18
TGs (mg/dL)	151.80 ± 7.17	185.60 ± 16.21^{a}	131.00 ± 5.58	136.33 ± 8.03

Results are means ± s.e.m. of *n* rats per group. $^{a}P<0.05$, $^{aa}P<0.01$ and $^{aaa}P<0.001$ *vs* the sedentary group (control); $^{b}P<0.05$, $^{bb}P<0.01$ and $^{bbb}P<0.001$ *vs* the Swimming group without rhEPO (exercise: EX). ALT, alanine aminotransferase; AST, aspartate aminotransferase; rhEPO, recombinant human erythropoietin; TGs, triglycerides; Total-c: total cholesterol

Table III – Effects of rhEPO on BP, HR and tissue trophy indexes in chronic exercise

Parameters	Sedentary		Swimming	
	Control (n=7)	rhEPO (n=7)	EX (n=7)	EX+rhEPO (n=7)
Blood Pressure and HR				
SBP (mmHg)	116.60 ± 0.70	120.00 ± 1.53^{a}	123.92 ± 1.38^{a}	136.67 ± 1.08^{bbb}
DBP (mmHg)	94.60 ± 1.00	110.67 ± 0.88^{aa}	108.33 ±1.34^{aa}	123.22 ± 2.04^{bbb}
MBP (mmHg)	100.10 ± 0.70	113.67 ± 0.88^{a}	113.25 ± 0.99^{a}	127.33 ± 1.62^{bbb}
HR (beats/min.)	341.30 ± 3.00	407.33 ± 13.93	394.58 ± 8.66	418.44 ± 6.57^{b}
Body and Tissue Weights				
BW (Kg)	0.51 ± 0.01	0.48 ± 0.10	0.46 ± 0.10^{a}	0.46 ± 0.09
HW (g)	1.19 ± 0.03	1.31 ± 0.05^{a}	1.23 ± 0.03	1.40 ± 0.03^{bb}
LVW (g)	0.57 ± 0.02	0.42 ± 0.02^{aaa}	0.58 ± 0.02	0.62 ± 0.02
KW (g)	1.42 ± 0.05	1.24 ± 0.04^{aa}	1.23 ± 0.06^{aa}	1.33 ± 0.04
LW (g)	16.78 ± 0.52	15.27 ± 0.43^{a}	15.62 ± 0.61	15.42 ± 0.38
AW (g)	0.057 ± 0.002	0.054 ± 0.01	0.069 ± 0.009	0.083 ± 0.008
MW(g)	23.57 ± 0.80	20.47 ± 1.1^{a}	22.46 ± 0.47	22.39 ± 0.83
Tissue Trophy Indexes				
HW/BW (g/kg)	2.35 ± 0.05	2.72 ± 0.10^{a}	2.65 ± 0.10	3.06 ± 0.16^{b}
LVW/HW (g/kg)	0.48 ± 0.02	0.38 ± 0.02^{aaa}	0.52 ± 0.01	0.45 ± 0.01^{b}
LVW/BW (g/kg)	1.12 ± 0.06	0.89 ± 0.03^{aa}	1.32 ± 0.09^{a}	1.38 ± 0.04
KW/BW (g/kg)	2.86 ± 0.09	2.56 ± 0.05^{a}	2.66 ± 0.13	2.82 ± 0.10
LW/BW (g/kg)	32.92 ± 0.57	31.72 ± 0.59	33.65 ± 1.15	34.34 ± 0.65
AW/BW (g/kg)	0.108 ± 0.01	0.114 ± 0.02	0.148 ± 0.02^{a}	0.175 ± 0.02
MW/BW (g/kg)	44.90 ± 0.91	45.06 ± 0.31	47.75 ± 0.59^{aa}	46.10 ± 0.46

Results are means ± s.e.m. a $p< 0.05$, aa – $p< 0.01$ and aaa – $p< 0.001$ *vs* the control and b $p< 0.05$, bb – $p< 0.01$ and bbb – $p< 0.001$ *vs* the EX group without rhEPO. AW, adrenals weight; BW: body weight; DBP, diastolic blood pressure; HR, heart rate; HW, heart weight; KW, kidney weight; LVW, left ventricle weight; LW, liver weight; MBP, mean blood pressure; MW, muscle weight; SBP, systolic blood pressure

Sympathetic and serotonergic measures

The rhEPO-treated rats presented a trend to higher values of plasma NA and AD vs control, together with lower platelet contents of both catecholamines and of NA in adrenals. Exercised rats also showed a trend to higher concentration of plasma NA and significantly higher ($P<0.001$) of platelet NA and AD, as well as lower content in the adrenals, which was statistically significant for AD ($P<0.05$). In the EX+rhEPO rats, however, the plasma NA and AD were significantly ($P<0.05$) higher when compared with EX group, which was accompanied by lower platelet AD content and a trend to higher concentration of this amina in adrenals and brain (Table IV). Concerning serotoninergic measures, while plasma 5-HT values were lower ($P<0.01$) in rhEPO rats vs control, 5-HIAA levels were higher ($P<0.01$). Similar pattern was found for the EX animals ($P<0.05$). The platelet levels showed an inverse profile. In the rats under exercise and rhEPO treatment, the plasma 5-HT ($P>0.001$) and 5-HIAA ($P<0.05$) concentrations were sub-

stantially higher than those observed in EX animals and the platelet and brain levels were maintained identical to those of the EX rats (Table IV).

Serum inflammatory profile and redox status markers

In the rhEPO rats there was significantly (P<0.05) higher values of TNF-α and TGF-β1 vs control. No significant changes were obtained for the EX group vs control. In the EX+rhEPO group, excepting the higher (P<0.05) values of TGF-β1 vs the EX group, there was no further differences between the groups (Table V).

In the rhEPO-treated rats (rhEPO group) there was an antioxidant effect, with an increment in TAS (P<0.001) and a decrease in 3-NT content (P<0.05). Similar pattern was found for the EX group vs control, particularly due to a decrease in serum MDA (P<0.05). However, in the rats of the EX+rhEPO group, the effect was pro-oxidant, with a trend to lower levels of TAS and higher of MDA, MDA/TAS and 3-NT vs the EX (Table V).

DISCUSSION

The rationale for its use in sport, as doping, is, thus, based on the increased oxygen capacity it provides, due to augmented erythropoietic stimulation[3]. Since rhEPO became available as an erthropoiesis-stimulating drug, its abusing use by athletes of endurance aerobic sports has been speculated and studied[2,5,6]. rhEPO doping remains one of the

Table IV – Effects of rhEPO on peripheral and central catecholamine and serotonin measures

Parameters	Sedentary		Swimming	
	Control (n=7)	rhEPO (n=7)	EX (n=7)	EX+rhEPO (n=7)
Catecholamines Measures				
NA – Plasma (ng/ml)	3.71 ± 0.60	4.81 ± 0.37	5.10 ± 0.96	9.32 ± 1.43^{b}
Platelet (ng/ml)	4.54 ± 0.61	0.60 ± 0.08^{aaa}	8.02 ± 0.68 ^{aaa}	7.01 ± 0.47
Adrenals (µg/g)	164.1 ± 8.0	130.4± 9.6^{a}	149.8 ± 15.8	133.8 ± 7.9
Brain (ng/g)	0.20 ± 0.004	0.18 ± 0.007	0.21 ± 0.008	0.19 ± 0.008
AD – Plasma (ng/ml)	1.48 ± 0.21	1.52 ± 0.06	1.04 ± 0.09	1.96 ± 0.18^{b}
Platelet (ng/ml)	0.69 ± 0.04	0.36 ± 0.08^{aa}	9.15 ± 2.26 ^{aaa}	0.50 ± 0.09^{bbb}
Adrenals (µg/g)	626.0 ± 47.6	602.0 ± 66.7	433.1 ± 24.6^{a}	579.4 ± 40.6
Brain (ng/g)	2.03 ± 0.09	2.38 ± 0.18	1.79 ± 0.25	2.57 ± 0.15 ^{b}
Serotonergic Measures				
5-HT – Plasma (ng/ml)	18.56 ± 1.46	5.82 ± 0.60^{aaa}	11.08 ± 0.65^{a}	30.07±4.45^{bbb}
Platelet (ng/ml)	556.7 ± 40.9	830.0±27.2^{aaa}	1610.8±55.1^{aaa}	1640.4±39.6
Brain (µg/g)	0.25 ± 0.01	0.30 ± 0.01^{a}	0.24 ± 0.01	0.22 ± 0.01
5-HIAA – Plasma (ng/ml)	11.53 ± 0.93	17.56 ±1.20^{aa}	18.00 ± 2.94^{a}	25.07 ± 2.38^{b}
Platelet (ng/ml)	3.92 ± 0.24	2.74 ± 0.18^{aa}	2.99 ± 0.22^{a}	3.68 ± 0.30
Brain (µg/g)	0.13 ± 0.004	0.12 ± 0.007	0.13 ± 0.005	0.13 ± 0.006

Results are means ± s.e.m. of *n* rats per group. ^{a}P<0.05, ^{aa}P<0.01 and ^{aaa}P<0.001 *vs* the sedentary group (control); ^{b}P<0.05, ^{bb}P<0.01 and ^{bbb}P<0.001 *vs* the Swimming group without rhEPO (exercise: EX). A, adrenaline; NA, noradrenaline; rhEPO, recombinant human erythropoietin; 5-HT, 5-hydroxy-tryptamine; 5-HIAA, 5-hydroxyindoleacetic acid

Table V – Effects of rhEPO on serum inflammatory profile and redox status markers

Parameters	Sedentary		Swimming	
	Control (n=7)	rhEPO (n=7)	EX (n=7)	EX+rhEPO (n=7)
Inflammatory Markers				
CRP (µg/mL)	26.68 ± 0.88	24.82 ± 0.76	24.22 ± 1.06	25.77 ± 1.21
IL-1β (pg/mL)	25.55 ± 1.69	25.95 ± 0.75	25.84 ± 1.38	23.96 ± 1.27
IL-2 (pg/mL)	44.67 ± 7.48	39.59 ± 4.75	51.48 ± 4.11	59.08 ± 3.76
TNF-α (pg/mL)	12.13 ± 0.65	14.18 ± 0.79[a]	12.62 ± 0.76	12.69 ± 0.59
TGF-β1 (pg/mL)	315.2 ± 13.2	380.2 ± 18.8[a]	317.8 ± 15.1	375.7 ± 23.5[b]
Redox Status				
MDA (µmol/L)	0.40 ± 0.02	0.38 ± 0.04	0.30 ± 0.02[a]	0.34 ± 0.01
TAS (mmol/L)	0.24 ± 0.01	0.36 ± 0.03[aaa]	0.25 ± 0.01	0.22 ± 0.01
MDA/TAS (10-3)	1.76 ± 0.16	1.13 ± 0.23[a]	1.27 ± 0.09[a]	1.53 ± 0.05
3-NT (nmol/L)	42.42 ± 8.25	25.02 ± 3.58[a]	37.96 ± 7.31	42.26 ± 6.90

Results are means ± s.e.m. of *n* rats per group. [a]*P*<0.05, [aa]*P*<0.01 and [aaa]*P*<0.001 *vs* the sedentary group (control); [b]*P*<0.05, [bb]*P*<0.01 and [bbb]*P*<0.001 *vs* the Swimming group without rhEPO (exercise: EX). CRP, C-reactive protein; IL-1β, interleukin 1β; IL-2, interleukin 2; MDA, malondialdehyde; rhEPO, recombinant human erythropoietin; TAS, total antioxidant status; TGF-β1, transforming growth factor β1; TNF-α, tumour necrosis factor α; 3-NT, 3-nitrotyrosine

negative highlights of world sport, with recurrent news about the distortion of sport values and ethics, by athletes, that, desperate to enhance their performance, try, illegally, to improve oxygen delivery to the muscles by using rhEPO[2,14,15]. In endurance sports, such as long-distance running, cycling and skiing, performance relies on an adequate O_2-supply to the heart and skeletal muscle. Hence, the rate of maximal O_2-uptake is an important determinant of aerobic physical power. However, athletes who abuse rhEPO seem to consider only the benefit to performance and ignore the short and long-term side-effects. There is a suspicion that rhEPO-induced erythrocytosis caused the death of about 20 world-class Dutch and Belgian Cyclists, although this was never proven[8,9], probably due to the lack of methodological capacity to distinguish between the endogenous and the recombinant EPO as well as due the lack of knowledge concerning the mechanisms underlying the side-effects of rhEPO. When Lasne and de Ceaurriz[4] were able to separate and distinguish by electrophoresis the endogenous and the rhEPO in human urine, the scandal of rhEPO use in sports was revealed, and the research and medical community was able to alert for the high health risks for the athletes.

The main risks of erythrocytosis (Hct>0.55 l/l) include heart failure, myocardial infarction, seizures, peripheral thromboembolic events and pulmonary embolism. Endurance athletes are at increased risk during the competition, if their blood viscosity increases further due to the great loss of fluid associated with sweating[6,8,9,15]. The relationship between hematocrit and other hemorheological parameters and performance was previously studied, particularly in order to evaluate the paradox of hematocrit in exercise physiology[16-18], which is viewed as the discrepancy between the higher performance in artificially increased hematocrit, due

to autotransfusion or rhEPO doping, versus the clear negative correlation between hematocrit and fitness in normal conditions, due to training-induced "autohemodilution"[16]. According to Brun *et al.*, in highly trained professional footballers, the physiological values of hematocrit vary between 36 and 48%[16]. Furthermore, there was a higher aerobic capacity in "low" hematocrit (<40%) subjects, while in the higher hematocrits (>44.6%) ones there was a trend to increased viscosity[16].

The consequences of physical exercise on the EPO concentrations have been poorly investigated. However, according to some data, exercise practise is able to reduce the serum EPO levels[19]. In a study with marathon athletes under rhEPO treatment, serum EPO concentrations were increased after both 3 and 31 hrs after exercise, but were unchanged immediately after the end of running[20]. In our study, the rats under chronic exercise practice and rhEPO treatment showed several markers of increased cardiovascular/thromboembolic risk. The increased RBC count vs the EX group without rhEPO treatment was confirmed, as expected. This was accompanied by development of hypertension and tachycardia. Increased blood pressure is a common feature in patients and athletes under rhEPO treatment[6,8,9,21], and might result both from increased blood viscosity and loss of hypoxia-induced vasodilatation. rhEPO treatment was also able to promote heart hypertrophy, which might be due to the blood hyperviscosity and could be viewed as a need to ensure proper blood circulation to peripheral tissues. Increased tachycardia might be explained by the increment in sympathetic activity, revealed by the higher values of plasma noradrenaline and adrenaline concentrations. This effect of rhEPO was previously documented, namely on hemodialyzed patients under rhEPO therapy[22]. Furthermore, there was an increment in plasma serotonergic measures, which might result from platelet overactivation, thus releasing the granule contents. The increased platelet reactivity was reported by others[7], and is in favour of an increased vascular reactivity, blood pressure and thromboembolic complications.

rhEPO has been successfully used in anaemic patients to correct their anaemia. However, its effects on non-hematopoietic cells and tissues, such as the brain and the heart, suggested new important insights to its use in other pathological conditions, such as the ischemia-reperfusion, heart failure and neurodegenerative diseases[23-28]. The rationale for its potential use in those disorders is based on its antioxidant, anti-apoptotic and anti-inflammatory properties, already known as "pleiotrophic actions"[29-32]. In our study, both the rhEPO treatment, *per se*, and the exercise practice have demonstrated a beneficial effect on redox status markers. Therefore, rhEPO alone has promoted a significant increment in serum antioxidant capacity (TAS), together with a reduction of 3-nitrotyrosine content, a marker of peroxynitrite formation, which is in agreement with its action on other pathological circumstances[29-32]. Swimming rats also demonstrated antioxidant effects, given by the reduction of serum lipid peroxidation (MDA). However, rhEPO use in rats

under chronic aerobic exercise favoured oxidative stress, viewed by the trend to higher values of serum redox status markers (MDA and 3-NT) and lower of antioxidant capacity (TAS). These results, in view of the deleterious effect of ROS, might represent a clear increased cardiovascular risk.

The suggested serum pro-oxidative pattern was accompanied by non-significant changes of all serum inflammatory markers, excepting the significant increment in TGF-β1, when compared with the exercised rats without rhEPO treatment. While the increase in the proliferation marker TGF-β1 might eventually explain the heart hypertrophy, the other non-significant results suggest that the deleterious effect of rhEPO in the rats under exercise is not mainly a consequence of an exacerbated oxidative stress and inflammation, but is primarily a mechanic reaction as a result of increased blood viscosity. Therefore, the increased RBC will originate blood pressure and heart and left hypertrophy in order to improve the ability to transport the high-viscose blood, and the oxygen it carries, to peripheral tissue. The tachycardia further needed is most certainly originated by sympathetic overactivation. Thus, instead of specific effects of rhEPO on cellular/molecular mechanisms related to inflammation and oxidative stress pathways, the high cardiovascular and thromboembolic risk seems to be due to a physiological response accompanied by growth factors increment and cardiac hypertrophy, which results in an undoubted life risk.

Our findings are in agreement with other studies, both in humans and animals under rhEPO treatment. In end stage chronic kidney disease patients, for example, rhEPO is able to correct the associated anemia but there is hematocrit increment, often associated with hypertension, thromboembolism and higher morbidity and mortality[33]. In mice transgenic for EPO, the increased hematocrit was linked with left and right ventricular hypertrophy and cardiac oedema, as well as with a reduced life expectancy[34]. Thus, erythrocytosis seems to increase the risk for myocardial infarction and stroke, which was observed in our experimental model of rhEPO sports doping in rats under aerobic chronic exercise and rhEPO treatment.

In conclusion, in this animal model of doping, rhEPO use, in situations of chronic/regular physical exercise, promotes not only the expected increased hematocrit, but also other marked modifications in circulatory, inflammatory and metabolic parameters, that may be expected to be deleterious. Thus, the experimental results of Ht in the EX+rhEPO group may support observations in athletes submitted to erythropoietin doping, which might be submitted to a serious cardiovascular and even live risk, which might be known and believed by all sports authorities and in particular by them and their physicians and themselves.

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