Erythropoietin Promotes Deleterious Cardiovascular Effects and Mortality Risk in a Rat Model of Chronic Sports Doping

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Abstract Athletes who abuse recombinant human erythropoietin (rhEPO) consider only the benefit to performance and usually ignore the potential short and long-term liabilities. Elevated haematocrit and dehydratation associated with intense exercise may reveal undetected cardiovascular risk, but the mechanisms underlying it remain to be fully explained. This study aimed to evaluate the cardiovascular effects of rhEPO in rats under chronic aerobic exercise. A ten week protocol was performed in four male Wistar rat groups: control—sedentary; rhEPO—50 IU kg^{-1}, 3 times/wk; exercised (EX)—swimming for 1 h, 3 times/wk; EX + rhEPO. One rat of the EX + rhEPO group suffered a sudden death episode during the week 8. rhEPO in trained rats promoted erythrocyte count increase, hypertension, heart hypertrophy, sympathetic and serotonergic overactivation. The suddenly died rat's tissues presented brain with vascular congestion; left ventricular hypertrophy, together with a "cardiac-liver", suggesting the hypothesis of heart failure as cause of sudden death. In conclusion, rhEPO doping in rats under chronic exercise promotes not only the expected RBC count increment, suggesting hyperviscosity, but also other serious deleterious cardiovascular and thromboembolic modifications, including mortality risk, which might be known and assumed by all sports authorities, including athletes and their physicians.

Introduction

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].

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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, compared to other sports 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 cardiovascular effects of rhEPO treatment on rats under chronic aerobic exercise; we also studied the probable cause of sudden death occurred in one rat under exercise practice and rhEPO treatment.

Materials and Methods

Animals and Experimental Protocol

Male Wistar rats (Charles River Laboratories Inc., Barcelona, Spain), weighting 220–250 g, were maintained in an air conditioned room (22–24°C) 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 no 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 ($n = 7$ 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 h, 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 ± 1°C, for 10 weeks. Excepting one animal of the EX + rhEPO group, which suffered a sudden death episode during an exercise session (week 8), all the animals have completed the 10-week protocol.

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 anaesthesia with a 2 mg kg^{-1} BW of a 2:1 (v:v) 50 mg ml^{-1} 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 20°C) to obtain platelet-rich plasma (PRP), which was then centrifuged (730 g for 10 min. at 20°C) to obtain the platelet pellet and poor platelet plasma (PPP).

Tissues

The rats were killed 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 haematoxylin–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, urea 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 protocol [11], using appropriate standards (Sigma Chemical Co., St. Louis, MO, USA) 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 protocol [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 (two-way) ANOVA and Fisher's PLSD test. Significance was accepted at P less than 0.05.

Results

General Characterization: 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 1).

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 (Table 1). The exercised rats demonstrated no relevant changes concerning haematological data, excepting higher MCHC versus 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 versus the EX rats without rhEPO therapy (Table 1). Platelet count and PCT were unchanged between groups, only demonstrating a trend to higher values in this group (data not shown).

Effect of rhEPO in Renal and Liver Function and Lipid Profile in Chronic Exercise

Urea content was lower ($P < 0.05$) in the rhEPO group versus control, without significant changes on creatinine and uric acid. Exercised rats also presented significantly lower values of urea ($P < 0.05$) and uric acid ($P < 0.01$).

Table 1 General characterization: serum EPO concentration and haematological 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
Haematological data				
RBC count ($\times 10^{12}$/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

Results are means ± s.e.m. of *n* rats per group

EPO erythropoietin; *Hb* haemoglobin; *MCH* mean corpuscular haemoglobin; *MCHC* mean corpuscular haemoglobin concentration; *MCV* mean corpuscular volume; *RBC* red blood cell and *rhEPO* recombinant human erythropoietin

[a] $P < 0.05$, [aa] $P < 0.01$ and [aaa] $P < 0.001$ versus the sedentary group (control)

[b] $P < 0.05$, [bb] $P < 0.01$ and [bbb] $P < 0.001$ versus the Swimming group without rhEPO (exercise: EX)

Table 2 Effects of rhEPO on renal and 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

ALT alanine aminotransferase; *AST* aspartate aminotransferase; *rhEPO* recombinant human erythropoietin; *TGs* triglycerides and *Total-c* total cholesterol

[a] $P < 0.05$, [aa] $P < 0.01$ and [aaa] $P < 0.001$ versus the sedentary group (control)

[b] $P < 0.05$, [bb] $P < 0.01$ and [bbb] $P < 0.001$ versus the Swimming group without rhEPO (exercise: EX)

This reduction was prevented in the rats under exercise and rhEPO treatment (EX + rhEPO; Table 2).

Serum AST was unchanged between groups, but there was a higher value of ALT in the rhEPO and in the EX rats versus the control. The change in the EX rats was not prevented by concomitant rhEPO treatment (Table 2).

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 2).

RhEPO Promotes/Aggravates Hypertension, Tachycardia and Cardiac Hypertrophy in Chronic Exercise

Blood pressure (SBP, DBP and MBP) and HR values were higher in the rhEPO 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 ($P < 0.05$; Fig. 1). Body weight showed a lower value ($P < 0.05$) in EX rats (0.46 ± 0.10 kg) versus control (0.51 ± 0.01 kg), without further changes between groups. HW and HW/BW were significantly higher

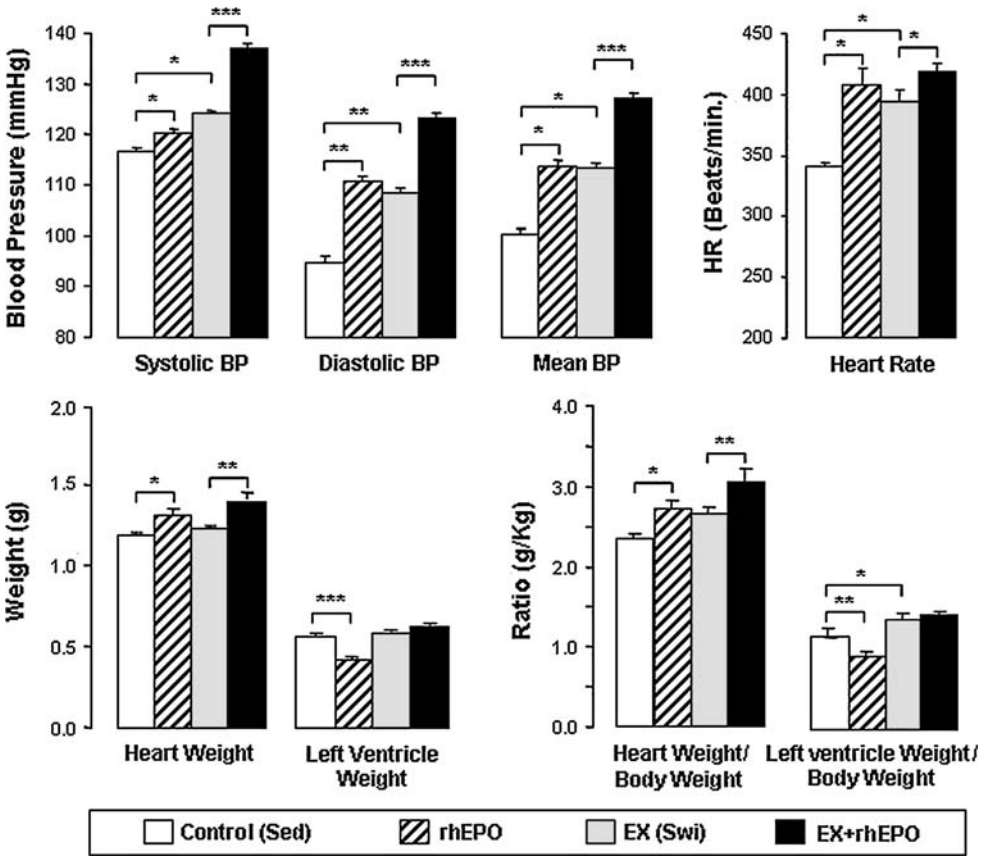


Fig. 1 Effects of rhEPO on blood pressures (systolic, diastolic and mean BP), heart rate and heart and left ventricle weights and trophy indexes in the rats of the 4 groups under study for 10 weeks: control (sedentary: Sed), rhEPO (50 IU/kg s.c., 3 times/wk; exercised (EX), which swam for 1 h, 3 times/wk and EX + rhEPO. Results are means ± s.e.m. of 7 rats per group. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$

in rhEPO group versus 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 (Fig. 1). Concerning the other tissues, the changes to report were in the rhEPO group, there was a significant reduction in ($P < 0.05$) KW/BW (2.56 ± 0.05 g kg^{-1}) versus the control (2.86 ± 0.09 g kg^{-1}), while in the rats under exercise, there were higher values of AW/BW (0.148 ± 0.02 g kg^{-1}; $P < 0.05$) and MW/BW (47.75 ± 0.59 g kg^{-1}; $P < 0.01$) versus the control (0.108 ± 0.01 and 44.90 ± 0.91, respectively).

RhEPO Promotes Peripheral Sympathetic and Serotonergic Overactivation in Chronic Exercise

The rhEPO-treated rats presented a trend to higher values of plasma NA and AD versus 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 ($P < 0.001$) concentration 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 amine in adrenals and brain (Table 3). Concerning serotoninergic measures, while plasma 5-HT values were lower ($P < 0.01$) in rhEPO rats versus 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 substantially higher than those observed in EX animals, and the platelet and brain levels were maintained identical to those of the EX rats (Table 3).

Effect of rhEPO in Serum Inflammatory Profile and Redox Status Markers in Chronic Exercise

In the rhEPO rats, there were significantly ($P < 0.05$) higher values of TNF-α and TGF-β1 versus control. No significant changes were obtained for the EX group versus control. In the EX + rhEPO group, excepting the higher ($P < 0.05$) values of TGF-β1 versus the EX group, there was no further differences between the groups (Table 4).

In the rhEPO-treated rats (rhEPO group), there was an antioxidant effect, with an increment in TAS ($P < 0.001$)

Table 3 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 adrenaline; *NA* noradrenaline; *rhEPO* recombinant human erythropoietin; *5-HT* 5-hydroxy-tryptamine; *5-HIAA* 5-hydroxyindoleacetic acid

[a] $P < 0.05$, [aa] $P < 0.01$ and [aaa] $P < 0.001$ versus the sedentary group (control)

[b] $P < 0.05$, [bb] $P < 0.01$ and [bbb] $P < 0.001$ versus the Swimming group without rhEPO (exercise: EX)

Table 4 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

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 and *3-NT* 3-nitrotyrosine

[a] $P < 0.05$, [aa] $P < 0.01$ and [aaa] $P < 0.001$ versus the sedentary group (control)

[b] $P < 0.05$, [bb] $P < 0.01$ and [bbb] $P < 0.001$ versus the Swimming group without rhEPO (exercise: EX)

and a decrease in 3-NT content ($P < 0.05$). Similar pattern was found for the EX group versus control, particularly due to a decrease in serum MDA ($P < 0.05$). However, in the rats of the EX + rhEPO group, no relevant changes were encountered for the redox status markers, being the values identical to those of the control group (Table 4).

Histomorphological Analysis of Tissues from the Suddenly Died Rat Under Exercise and rhEPO Treatment

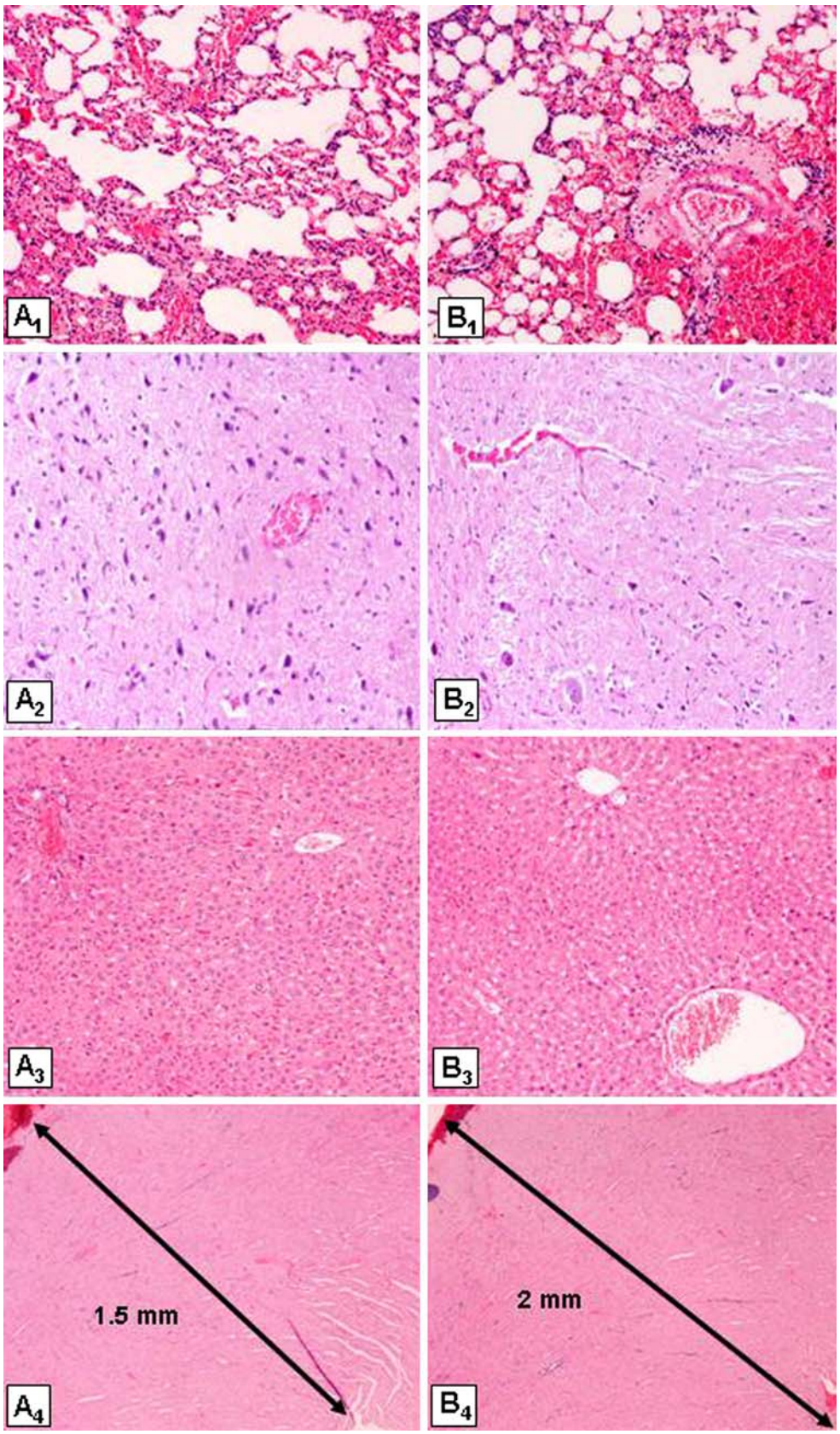
In the week 8 of exercise practice, one rat of the EX + rhEPO group suffered a sudden death episode in the initial 15 min of the swimming session. All efforts with reanimation procedures were, unfortunately, useless. As the blood collection was compromised due the time taken in the animal assistance, we removed and weighted the kidneys, the lungs, the brain, the heart and the liver to assess the possible cause of death. The rat heart weight was 1.82 g, and the heart/body weight ratio was 4.04 g kg^{-1}, significantly hypertrophic when compared with the values of the EX group (1.23 ± 0.06 g and 2.66 ± 0.13 g kg^{-1}, respectively), demonstrating the tremendous effort of the heart to maintain its functions. The histomorphological studies provided the following results, when compared with the normal lungs, brain, liver and left ventricle (Fig. 2A1–A4, respectively): the kidneys from the suddenly died rat showed eosinophilia and congestion (data not shown); the lungs showed signs of blood congestion, alveolar haemorrhage and anoxia, without markers of drowning (Fig. 2B1); the brain tissue from the suddenly died rat demonstrated vascular congestion (Fig. 2B2); the liver showed centre-lobular congestion and signals of "cardiac liver", probably due to the heart failure (Fig. 2B3); there was LVH (2.0 mm^3) when compared with the values of the control group (1.5 mm^3) and deregulation of cardiac fibres, suggesting the hypothesis of heart failure (eventually ventricular fibrillation) as cause of death (Fig. 2B4).

Discussion

The rationale for the use of erythropoietin in sports as doping is based on the increased oxygen capacity it provides, due to augmented erythropoietic stimulation (3). Since rhEPO became available as an erythropoiesis-stimulating drug, its abuse by athletes of endurance aerobic sports has been speculated and studied [2, 5, 6]. rhEPO doping remains one of the negative highlights of world sport, with recurrent news about the distortion of sport values and ethics by athletes, who desperate to enhance their performance, trying illegally to improve oxygen delivery to the muscles by using rhEPO [2, 14, 15]. In endurance sports, such as long-distance running and 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]. Interestingly, some deaths allegedly caused by rhEPO have not occurred during exercise but during periods of physical inactivity, suggesting that the deleterious effects are prolonged.

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 [16]. In a study with marathon athletes under rhEPO treatment, serum EPO concentrations were increased after both 3 and 31 h after exercise but were unchanged immediately after the end of running [17]. In our study, the rats under chronic exercise practice and rhEPO treatment showed several markers of increased cardiovascular/thromboembolic risk. The increased RBC count versus the EX group without rhEPO treatment was confirmed, as expected. This was accompanied by the development of hypertension and tachycardia. Increased blood pressure is a common feature in patients and athletes under rhEPO treatment [6, 8, 9, 18] 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, suggested by the RBC count increment, 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 haemodialyzed patients under rhEPO therapy [19]. 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.


A1
B1
A2
B2
A3
B3
1.5 mm
A4
2 mm
B4

◀ **Fig. 2** Histomorphological H&E staining pictures from the lung ($_1$), the brain ($_2$), the liver ($_3$) and the left ventricle ($_4$) from the control rats (**A**) when compared with those of the suddenly died rat of the EX + rhEPO group (**B**). During the week 8 of study, one rat of the EX + rhEPO group (exercise and rhEPO treatment) suffered a sudden death episode. As the blood collection was compromised due to the time taken in the animal assistance, we removed and weighted several tissues to assess the possible cause of death. The histomorphological studies provided the following results, when compared with the normal lungs, brain, liver and left ventricle (*A1–A4*, respectively): lungs with signs of blood congestion, alveolar haemorrhage and anoxia, without markers of drowning (*B1*); brain with vascular congestion (*B2*); liver showing centre-lobular congestion and signals of "cardiac liver" (*B3*) and left ventricular hypertrophy (2.0 mm^3) when compared with the values of the control group (1.5 mm^3), and deregulation of cardiac fibres, suggesting the hypothesis of heart failure (eventually ventricular fibrillation) as cause of death (*B4*)

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 ischaemia–reperfusion, heart failure and neurodegenerative diseases [20–25]. 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" [26–29]. 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 in 3-nitrotyrosine content, a marker of peroxynitrite formation, which is in agreement with its action on other pathological circumstances [26–29]. Swimming rats also demonstrated antioxidant effects, given by the reduction of serum lipid peroxidation (MDA). In the rats under exercise and rhEPO treatment, no relevant changes were encountered for the redox status markers, being the values identical to those of the control animals.

No significant changes were encountered in the serum inflammatory markers, excepting the significant increment in TGF-β1, a powerful profibrogenic cytokine, when compared with the exercised rats without rhEPO treatment. It is interesting to note that with exercise, there is adaptive left ventricular hypertrophy, which might be rendered pathologic (fibrosis) with rhEPO, creating substrate for heart failure and sudden death from arrhythmias.

All the changes reported for the EX + EPO rats seem to be in agreement with the sudden death episode occurred in one rat of the group, after 8 weeks of protocol, during the initial minutes of exercise. The anatomo-pathological tissue evaluation of the suddenly died rat demonstrated that there were no drowning signs in the lungs, but marked vascular congestion in the lungs, brain and liver. Furthermore, and even more relevant, there was some LVH and deregulation of cardiac fibres, together with a "cardiac liver", suggesting the hypothesis of heart failure as the cause of death, which is in agreement with the increased risk of cardio/cerebrovascular and thromboembolic events that the functional studies in the EX + EPO also indicate. Further studies will be important to assess the nature of the changes in myocardial structure encountered in the rhEPO rats and in particular in those suffering sudden dead episodes, in order to evaluate the influence on myocyte size, capillary density and reactive myocardial fibrosis, and its relationship with the deleterious effects and mortality risk.

Our findings are in agreement with other studies, in both humans and animals under rhEPO treatment. In end stage chronic kidney disease patients, for example, rhEPO is able to correct the associated anaemia, but there is haematocrit increment, often associated with hypertension, thromboembolism and higher morbidity and mortality [30]. In mice transgenic for EPO, the increased haematocrit was linked with left and right ventricular hypertrophy and cardiac oedema, as well as with a reduced life expectancy [31]. 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, rhEPO use, as doping, in situations of chronic/regular physical exercise, promotes not only the expected increased erythrocytosis, suggesting hyperviscosity, but also other serious deleterious cardiovascular and thromboembolic modifications, such as hypertension, heart hypertrophy, sympathetic and serotonergic overactivity. Thus, our experimental model of rhEPO sports doping demonstrates that athletes under similar conditions are submitted to a serious cardiovascular and even mortality 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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