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TOXICOLOGICAL EFFECTS OF RECOMBINANT HUMAN ERYTHROPOIETIN DOPING – CHRONIC VERSUS ACUTE AEROBIC EXERCISE

Abstract: *Aims:* Compare the toxicological effects of rhEPO on rats under chronic versus acute exercise.

Protocol: Male Wistar rat groups for the chronic exercise (swimming) – 10 wks of treatment: – control – sedentary (SED); – rhEPO – 50 IU/Kg/wk; swimming (EX) – 1 hr, 3 times/wk; EX+EPO. For the extenuating exercise (rhEPO given for 3 wks prior to exercise): – swimming (Swi); – Swi+EPO (50 IU/Kg/wk); – running (Run); – Run+EPO. Blood and tissue samples were assessed for: haematology, catecholamine and serotoninergic measures and redox status.

Results: The chronic EX+EPO rats showed higher values of RBC, Htc and Hb vs EX and vs Swi+EPO of the acute sessions. Both chronic and acute swimming showed a remarkable sympathetic and serotonergic activation. rhEPO treatment in chronic training has promoted oxidative stress, in contrast with the antioxidant effect on Swi and Run of acute exercises.

Conclusions: rhEPO doping is more deleterious in rats mimicking high-performance athletes (chronic training) than in occasional consumers (acute sessions), due to increased CV risk.

Keywords: rhEPO; doping; toxicological effects; chronic *vs* acute aerobic exercise; haemogram; sympathetic and serotoninergic activation; oxidative stress.

Introduction

Erythropoietin (EPO) is a circulating glycosylated protein hormone, synthesized mainly in the kidneys, that is the primary regulator of RBC formation (1). The production of recombinant human erythropoietin (rhEPO), which has been widely used for correction of anaemia, allowed many patients to resume their normal daily activities due to increased energy (2).

The rationale for the use of rhEPO in sport, as doping, is based on the increased O2 capacity it provides, due to augmented erythropoietic stimulation (3). 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).

Athletes who abuse rhEPO consider only the benefit to performance and usually ignore the potential short and long-term liabilities. Elevated Hct and dehydratation associated with intense exercise may reveal undetected CV risk in some athletes (7,8). Illegal and abusive utilization of this hormone has been found in both endurance and short-duration sports, which require distinct energetic sources, but the potential deleterious effects and mechanism underlying, remain to be fully elucidated.

This study intended to compare the toxicological effects of rhEPO treatment on rats under chronic vs acute exercise, as well as to assess the differences between two distinct modalities of extenuating exercise.

Material and methods

Animals and experimental protocol

Male Wistar rats (Charles River Lab., Spain), 220-250g, were maintained in appropriate conditioned: 22-24ºC; 60% humidity; 12-h dark-light cycles; standard rat chow (AO4, Panlab, Letica, Spain) and water *ad libitum*.

For the chronic exercise (swimming), after a period of adaptation of 2 week, 4 groups (n=8) were evaluated for 10 wks-treatment: control – sedentary (SED); rhEPO – 50 IU/Kg/wk beta-EPO Recormon®, Roche Pharm. (EPO); Exercised (EX) – swimming (1 hr, 3 times/wk); EX+EPO. The swimming rats were submitted to a 1 wk period of adaptation for minimizing the water stress (bath set at 35±1ºC). Sessions started with 15 min, increased 5 min each day until a 60 min continuous period was achieved.

For the acute exercise, the following groups were tested: swimming (Swi); Swi+EPO; running (Run) and Run+EPO. rhEPO (50 IU/Kg/wk) was given for 3 wks prior to the extenuating sessions. Running was performed in a treadmill (Letica LE 8706, Spain) at the velocity of 54 cm/s and an inclination of 15%. Both exercises were made without previous adaptation and until extenuation. Duration and/or distance were monitored.

Sample collection and preparation

Serum, plasma and platelets were obtained from the rat blood collected i.p. ketamine anesthesia at the end of treatments. Some tissues were excised and stored for further analysis: adrenals, left ventricle (LV) and brain in HClO4 and gastrocnenius muscle in liquid nitrogen.

Haematological data

Red blood cell (RBC) count, haematocrit (Hct), haemoglobin (Hb), platelets count, mean platelet volume (MPV), platelet distribution width (PDW) and plaquetocrit (PCT) were assessed by using an automatic Coulter Counter® (Beckman Coulter Inc., USA).

Catecholamine and serotonin assay

Noradrenaline (NA) and adrenaline (A) concentrations in plasma, platelet, adrenals, left ventricle and brain, as well as plasma, platelet and brain 5-hydroxy-tryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) contents, were evaluated by HPLC-ED, according to previously described (9). Concentrations were expressed in ng/ml for plasma and platelets and in µg/g for adrenals, left ventricle and brain.

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 (9). MDA concentration was expressed as µmol/l in serum and as µmol/g tissue in muscle. Ferric reducing antioxidant potential (FRAP) assay was used to estimate serum and muscle total antioxidant status (TAS) (9).

Data Analysis

Results are presented as means ± s.e.m. Comparisons between groups were performed using one-way ANOVA and Fisher's test. Significance was accepted at p less than 0.05.

Results

Acute exercise performance

The animals from the EX group have swim for 50.67 ± 2.19 min, while the Swi+EPO rats have performed a longer swimming period (56.33±5.24 min). Similar rhEPO influence was found for the Run (38.67±2.19 min) and Run+EPO groups (43.00±6.11 min). In this assay, the distance of run was also higher in the rats under rhEPO treatment (678.33±75.08 m), when compared with those without rhEPO (662.67±30.69 m).

Haematological data (Table 1)

The most important findings were that rhEPO in the chronic exercise was able to increase RBC count (p<0.05), with a trend to increased Hct and Hb, while in the acute there was a significant reduction (p<0.01) of those parameters in the Swi protocol, with a trend to reduce in the Run. Platelet count showed a trend to increased values in the chronic Swi+EPO rats vs EX. Similar pattern, but significant (p<0.05), was found in the Swi+EPO of the acute exercise, contrasting with the trend to lower values in the Run extenuating sessions with rhEPO.

Catecholamine and serotonin measures (Table 2)

In the chronic Swi+EPO animals, there was an increment in plasma NA (p<0.05) and AD (p<0.01) contents, accompanied by a trend to NA reduction in adrenals and platelets and a significant decrease in the LV (p<0.001), together with a trend to AD increment in adrenals and a significant reduction (p<0.001) in platelets. An identical

plasma NA and AD (p<0.001) pattern of changes was found for the acute Swi, being the levels higher vs Run+rhEPO. The NA and AD increase in the acute Swi+EPO rats was accompanied by a reduction in platelet NA and by an increment in brain AD. In the acute Run+EPO, the main changes were a trend to higher values of NA in plasma and adrenals and reduction in platelets, together with a trend to lower values of AD in plasma and higher in platelets and adrenals.

While in the chronic training the changes were non-significant for 5-HT and 5-HIAA in the plasma, platelets and brain, in the Swi+EPO of the acute sessions there was an increment in plasma measures and a reduction in platelets. Similar pattern was found for Run+EPO vs Run.

Serum and muscle redox status (Table 3)

In serum samples, the redox status, evaluated by the MDA/TAS levels, increased (p<0.05) in the Swi+EPO chronic exercise, while there was a trend to a reduction in the Swi+EPO extenuating exercise. Similar pattern was found for the muscle assays, showing that rhEPO was pro-oxidant when given in chronic training conditions but antioxidant in acute. Concerning the extenuating exercises, the running is notoriously more oxidative than the swimming, but the rhEPO treatment demonstrated similar antioxidant profile, both in serum and muscle samples.

Discussion

The therapeutic use of rhEPO, particularly for the treatment of anaemia, allowed a significant reduction in the associated adverse effects and improved patient's quality of life (2). Unfortunately, some athletes and their coaches were eager to abuse rhEPO because it increases the O2 supply to muscles and boots performance in endurance sports, such as skiing, running and cycling (3). This led to a view among some athletes that to compete successfully doping with rhEPO was required, forgetting the increased health risk. The increased Hct above certain levels causes important side-effects, which includes hypertension (HT), heart failure, myocardial infarction, peripheral thromboembolic events and pulmonary embolism, as well as shorten lifespan (10,11).

Athletes are at increased risk during the competition, due to the blood hyperviscosity, further aggravated by the great loss of fluid associated with sweating (6-8). In the early 1990s, there was considerable speculation that doping with rhEPO was involved in the death of professional cyclists (8), some of them occurred during periods of physical inactivity, indicating that the deleterious effects remain after the competition. Abusive use of rhEPO might be viewed in both endurance and short-duration sports, which require distinct energetic sources, but the potential deleterious effects and mechanisms underlying remain to be fully elucidated. Our data confirmed that rhEPO promoted an augmented sports performance.

The consequences of physical exercise on the EPO concentrations have been poorly investigated. In a study with marathon athletes under rhEPO treatment, serum EPO levels increased after both 3 and 31 hrs after exercise, but were unchanged immediately after the end of running (12). In our study, rhEPO treatment was able to increase the

RBC, the Hct and Hb in chronic exercise, without significant changes in acute, or even with a decrease, particularly in swimming extenuating. Thus, prolonged rhEPO treatment (10 wks in chronic) seems to be able to promote important changes on erythropoiesis, while in short-term (3 wks prior acute) do not produce identical stimulation. This should have distinct implications in CV risk, with an expected hyperviscosity in chronic exercise with rhEPO use.

HT is one of the main deleterious effects of rhEPO therapy (13), but, apart from the blood hyperviscosity, the mechanisms underlying remain to be fully explained. Sympathetic nervous system (SNS) overactivity have been suggested as a possible explanation (14). In our study, swimming promoted more pronounced effects on catecholamines levels than running, confirming previous data from us (9). Both the chronic and the acute swimming exercise showed a remarkable sympathetic and serotonergic activation, which might be due to the cardio-respiratory involvement, favouring the CV risk.

Distinct types and intensities of exercise have been associated with different effects on oxidative stress. Regular training is able to promote antioxidant actions (15), while high intensity exercise or in non-adapted individuals might produce harmful effects, in a dual effect known as "exercise paradox". The final effect seems to depend, thus, on the intensity as well as on the type of protocol. rhEPO treatment have been associated with beneficial therapeutic effects on non-anaemic conditions, due to its cardio and neuroprotective actions, attributed to a pleiotropic action, such as its antioxidant ability (16). In our study, rhEPO was notoriously more deleterious when used in chronic conditions, demonstrating a pro-oxidative action, contrasting to its putative antioxidant effect when used in acute extenuating exercises. This effect might be due to the lower duration of rhEPO treatment prior to extenuating exercise session (3 wks), when contrasting with the 10 wks for the chronic exercise, as well as with particular characteristics of acute exercise. Therefore, extenuating exercise leads to important autonomic and haemodynamic adaptations that influence the CV system in order to maintain homeostasis in response to the increase of metabolic needs. This includes augment of cardiorespiratory responses to promote increase of O2 supply to peripheral tissues; SNS activation, which increases HR and cardiac output and, then, BP fluxes to peripheral tissues, particularly to the muscles that needs more energy to produce work (17). Under those conditions, rhEPO seem to be needed, playing thus an antioxidant effetc, contrasting with its deleterious pro-oxidant profile when used in prolonged and regular training condition, mimicking chronic rhEPO doping.

Conclusions

The effects of rhEPO doping in rats under exercise is notoriously more deleterious in circumstances that mimic high-performance athletes (chronic training) than in occasional consumers (acute sessions), particular due to increased cardiovascular risk.

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Parameters	CHRONIC EXERCISE (training)			
	Sedentary		Swimming	
	No rhEPO	With rhEPO	No rhEPO	With rhEPO
RBCs				
RBC count (x10^{12}/L)	7.31 ± 0.16	7.67 ± 0.08*	7.59 ± 0.15	8.23 ± 0.14*
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
Platelet				
Platelet count (x10^{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*	5.63 ± 0.14	5.85 ± 0.35
PDW (%)	16.85 ± 0.15	16.73 ± 0.18	16.37 ± 0.26	17.00 ± 0.10
Parameters	ACUTE EXERCISE (extenuating session)			
	Sedentary		Swimming	
	No rhEPO	With rhEPO	No rhEPO	With rhEPO
RBCs				
RBC count (x10^{12}/L)	8.11 ± 0.23	6.65 ± 0.21**	7.97 ± 0.20	7.96 ± 0.21
Hb (g/dL)	14.70 ± 0.12	13.05 ± 0.55**	15.17 ± 0.20	14.83 ± 0.03
Hct (%)	40.73 ± 0.42	35.80 ± 1.70**	42.67 ± 0.64	41.87 ± 0.62
Platelet				
Platelet count (x10^{9}/L)	993.3 ± 40.7	1223.5 ± 144.5*	929.0 ± 69.0	774.0 ± 18.2
PCT (%)	0.55 ± 0.01	0.64 ± 0.08	0.44 ± 0.06	0.46 ± 0.01
MPV (fL)	5.50 ± 0.17	5.25 ± 0.05	5.87 ± 0.47	5.93 ± 0.23
PDW (%)	16.17 ± 0.43	15.70 ± 0.00	16.47 ± 0.58	16.63 ± 0.46

Results are means ± s.e.m. *p<0.05, **p<0.01 and ***p<0.001 *vs* the No rhEPO group.

Table 1 – Effects of rhEPO on haematological data in chronic and acute exercise protocols

Parameters	CHRONIC EXERCISE (training)			
	Sedentary		Swimming	
	No rhEPO	With rhEPO	No rhEPO	With rhEPO
Catecholamines measures				
Plasma NA (ng/ml)	3.71 ± 0.60	4.81 ± 0.37	5.10 ± 0.96	9.32 ± 1.43
AD (ng/ml)	1.48 ± 0.21	1.52 ± 0.06	1.04 ± 0.09	1.96 ± 0.18
Platelet NA (ng/ml)	4.54 ± 0.61	0.60 ± 0.08***	8.02 ± 0.68	7.01 ± 0.47
AD (ng/ml)	0.69 ± 0.04	0.36 ± 0.08**	9.15 ± 2.26	0.50 ± 0.09
Adrenals NA (µg/g)	164.1 ± 8.0	130.4 ± 9.6*	149.8 ± 15.8	133.8 ± 7.9
AD (µg/g)	626.0 ± 47.6	602.0 ± 66.7	433.1 ± 24.6	579.4 ± 40.6
L.Ventricl. NA (µg/g)	0.14 ± 0.02	0.71 ± 0.05***	0.92 ± 0.04***	0.12 ± 0.02***
AD (µg/g)	0.02 ± 0.01	0.05 ± 0.01	0.15 ± 0.02***	0.13 ± 0.02***
Brain NA (µg/g)	0.20 ± 0.004	0.18 ± 0.007	0.21 ± 0.008	0.19 ± 0.008
AD (µg/g)	2.03 ± 0.09	2.38 ± 0.18	1.79 ± 0.25	2.57 ± 0.15*
Serotoninergic measures				
Plasma 5-HT (ng/ml)	18.56 ± 1.46	5.82 ± 0.60***	11.08 ± 0.65	30.07 ± 4.45***
5-HIAA (ng/ml)	11.53 ± 0.93	17.56 ± 1.20**	18.00 ± 2.94	25.07 ± 2.38*
Platelet 5-HT (ng/ml)	556.7 ± 40.9	830.0 ± 27.2***	1610.8 ± 55.1	1640.4 ± 39.6
5-HIAA (ng/ml)	3.92 ± 0.24	2.74 ± 0.18**	2.99 ± 0.22	3.68 ± 0.30
Brain 5-HT (ng/g)	0.25 ± 0.01	0.30 ± 0.01*	0.24 ± 0.01	0.22 ± 0.01
5-HIAA (ng/g)	0.13 ± 0.004	0.12 ± 0.007	0.13 ± 0.005	0.13 ± 0.006

Parameters	ACUTE EXERCISE (extenuating session)			
	Sedentary		Swimming	
	No rhEPO	With rhEPO	No rhEPO	With rhEPO
Catecholamines measures				
Plasma NA (ng/ml)	3.91 ± 0.85	5.29 ± 1.95	1.52 ± 0.33	2.29 ± 0.55
AD (ng/ml)	1.95 ± 0.33	4.60 ± 0.41**	1.76 ± 0.02	1.15 ± 0.29
Platelet NA (ng/ml)	4.41 ± 0.79	2.13 ± 0.37	2.68 ± 1.46	1.50 ± 0.36
AD (ng/ml)	1.09 ± 0.01	1.59 ± 0.30	2.01 ± 0.86	2.72 ± 0.46
Adrenals NA (µg/g)	188.3 ± 11.0	188.1 ± 96.7	96.9 ± 24.1	158.4 ± 16.1
AD (µg/g)	865.0 ± 107.6	778.5 ± 423.6	411.5 ± 117.4	645.0 ± 40.4
L.Ventricl. NA (µg/g)	0.48 ± 0.11	0.43 ± 0.07	0.48 ± 0.01	0.45 ± 0.05
AD (µg/g)	0.10 ± 0.03	0.11 ± 0.02	0.24 ± 0.03gg	0.16 ± 0.01i
Brain NA (µg/g)	0.16 ± 0.01	0.18 ± 0.03	0.15 ± 0.01	0.22 ± 0.02
AD (µg/g)	3.95 ± 0.06	10.35 ± 2.25*	7.57 ± 1.40	5.90 ± 1.57
Serotoninergic measures				
Plasma 5-HT (ng/ml)	85.97 ± 20.70	533.4 ± 60.7***	75.83 ± 39.12	27.31 ± 6.43
5-HIAA (ng/ml)	17.10 ± 0.71	21.72 ± 0.62	18.60 ± 2.75	21.62 ± 1.51
Platelet 5-HT (ng/ml)	1122.2 ± 135.9	727.6 ± 45.2	622.8 ± 266.6	528.8 ± 104.9
5-HIAA (ng/ml)	4.34 ± 0.74	0.65 ± 0.14*	4.75 ± 0.91	2.82 ± 0.54
Brain 5-HT (ng/g)	0.14 ± 0.04	0.21 ± 0.03	0.16 ± 0.01	0.18 ± 0.02
5-HIAA (ng/g)	0.25 ± 0.02	0.24 ± 0.03	0.27 ± 0.03	0.30 ± 0.06

Results are means ± s.e.m. *p<0.05, **p<0.01 and ***p<0.001 *vs* the No rhEPO group.

Table 2 – Effects of rhEPO on peripheral and central catecholamine and serotoninergic measures in chronic and acute exercise protocols

Parameters	CHRONIC EXERCISE (training)			
	Sedentary		Swimming	
	No rhEPO	With rhEPO	No rhEPO	With rhEPO
Serum redox status				
MDA (µmol/L)	0.40 ± 0.02	0.38 ± 0.04	0.30 ± 0.02*	0.34 ± 0.01
TAS (µmol/L)	0.24 ± 0.01	0.36 ± 0.03***	0.25 ± 0.01	0.22 ± 0.01
MDA/TAS	1.76 ± 0.16	1.13 ± 0.23*	1.27 ± 0.09*	1.53 ± 0.05
Muscle redox status				
MDA (µmol/L)	0.29 ± 0.03	0.36 ± 0.02	0.38 ± 0.04	0.47 ± 0.04*
TAS (µmol/L)	0.14 ± 0.003	0.12 ± 0.001	0.12 ± 0.003	0.12 ± 0.009
MDA/TAS	0.41 ± 0.04	0.63 ± 0.04**	0.58 ± 0.03*	0.73 ± 0.06*
Parameters	ACUTE EXERCISE (extenuating session)			
	Sedentary		Swimming	
	No rhEPO	With rhEPO	No rhEPO	With rhEPO
Serum redox status				
MDA (µmol/L)	0.29 ± 0.08	0.22 ± 0.13	0.29 ± 0.10	0.36 ± 0.06
TAS (µmol/L)	0.45 ± 0.14	0.65 ± 0.20	0.56 ± 0.13	0.71 ± 0.04
MDA/TAS	0.37 ± 0.02	0.28 ± 0.14	0.67 ± 0.33	0.53 ± 0.11
Muscle redox status				
MDA (µmol/L)	1.04 ± 0.31	0.73 ± 0.09	1.82 ± 0.61	1.47 ± 0.34
TAS (µmol/L)	1.36 ± 0.22	1.57 ± 0.34	1.04 ± 0.32	1.31 ± 0.20
MDA/TAS	0.84 ± 0.35	0.48 ± 0.10	1.89 ± 0.58	1.19 ± 0.33

Results are means ± s.e.m. *p<0.05, **p<0.01 and ***p<0.001 *vs* the No rhEPO group.

Table 3 – Effects of rhEPO on serum and muscle redox status markers in chronic and acute exercise protocols