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"Effects of recombinant human erythropoietin high mimicking abuse doses on oxidative stress processes in rats"

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ABSTRACT

Although many studies highlight how long-term moderate dose of Recombinant Human Erythropoietin (rHuEPO) treatments result in beneficial and antioxidants effects, few studies take into account the effects that short-term high doses of rHuEPO (mimicking abuse conditions) might have on the oxidative stress processes. Thus, the aim of this study was to investigate the *in vivo* antioxidant activity of rHuEPO, administered for a short time and at high doses to mimic its sports abuse as doping. Male Wistar healthy rats (n = 36) were recruited for the study and were treated with three different concentrations of rHuEPO: 7.5, 15, 30 μg/kg. Plasma concentrations of erythropoietin, 8-epi Prostaglandin $F_{2\alpha}$, plasma and urinary concentrations of NOx were evaluated with specific assay kit, while hematocrit levels were analyzed with an automated cell counter. Antioxidant activity of rHuEPO was assessed analyzing the possible variation of the plasma scavenger capacity against hydroxylic and peroxylic radicals by TOSC (Total Oxyradical Scavenging Capacity) assay. Statistical analyses showed higher hematocrit values, confirmed by a statistically significant increase of plasmatic EPO concentration. An increase in plasma scavenging capacity against peroxyl and hydroxyl radicals, in 8-isoprostane plasmatic concentrations and in plasmatic and urinary levels of NO_X were also found in all the treated animals, though not always statistically significant. Our results confirm the literature data regarding the antioxidant action of erythropoietin administered at low doses and for short times, whereas they showed an opposite incremental oxidative stress action when erythropoietin is administered at high doses.

1. Introduction

Erythropoietin (EPO) is a glycoproteic plasmatic growing hormone secreted by the kidney in response to low levels of oxygen in the tissues. It stimulates the differentiation of progenitors cells into erythrocytes [1,2] and therefore increases the red blood cell mass for the maintenance of vascular oxygen homeostasis [3]. Human EPO was first purified in 1977 and cloned in 1985, which lead to the production of the recombinant human erythropoietin form (rHumanEPO, rHuEPO) [4].The introduction of rHuEPO in clinical practice, more than two decades ago, altered completely the management of patients with chronic kidney disease (CKD); in fact it is known to be widely used as therapeutical treatment for the anemia associated with chronic renal insufficiency. Moreover, the availability of rHuEPO has enhanced the lives of patients with chronic kidney disease, which is cause of anemia due to the inadequate production of EPO in kidney. However, clinical studies have demonstrated that rHuEPO is also effectiveness in various non-uremic conditions, such as anemia associated with onco-hematological disorders, prematurity, HIV infection and to reduce the exposure to allogeneic blood in surgical patients [5,6]. Concurrently its use in sports as doping has unfortunately spread.

The rHuEPO is one of the most widely used drugs to improve performance in sport by increasing the circulating red blood cells and the consequent oxygen delivery to muscles [7,27]. Sports authorities prohibited the use of rHuEPO in 1988. Athletes who abuse erythropoietin consider only the benefit to performance and usually ignore the potential short- and long-term liabilities [8,9,27]. The artificial increase in red blood cells count and

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hematocrit, further enhanced by dehydratation during prolonged exercise, predisposes to thromboembolic complications which might be connected to sudden death in sport practice [10].

However, since the mild 1990s, EPO has been shown to exert numerous extra-hematopoietic effects. One of them could be its antioxidant properties [11,19].

Experimental and biological studies showed conflicting results on antioxidant effects of the clinical use of erythropoietin. Katavetin et al. [14] suggested that this glycoprotein hormone may have antioxidant properties, having regard to its ability to attenuate free radical-mediated lipid peroxidation in patients when administered in its recombinant form (rHuEPO). Antioxidant activity of erythropoietin was also confirmed in other many clinical reports, in which EPO therapy could reduce plasma-oxidative stress in dialysis patients [11–17]. Rjiba-Touati et al. [17] showed that rHuEPO administration in pre-treatment condition protects rats against MMC-induced heart and renal oxidative stress and genotoxicity. In addition, Kumar et al. [5] demonstrated that treatment with erythropoietin (500 and 1000 IU/Kg i.p.) exerts a positive effect in memory deficits of mice through its multiple actions, including potential anti-oxidative effect. Otherwise, Rancourt et al. [19] showed that administration of EPO induces an increase of oxidative stress in uremic rats, speculating another role of erythropoietin.

In particular, although many studies highlight how long-term moderate dose of rHuEPO treatments result in beneficial and especially antioxidants effects, few studies take into account the effects that short-term high doses of rHuEPO (mimicking abuse conditions) might have on oxidative stress processes [32–41].

On the basis of what has just been elucidated, the purpose of this study was to observe the effects in animal models of rHuEPO, administered for a short time and at high doses to mimic its sports abuse as doping. In detail, the role of rHuEPO on the oxidative stress process in *vivo* was assessed by analyzing the possible variation of the plasma scavenger capacity against hydroxylic and peroxylic radicals by TOSC (Total Oxyradical Scavenging Capacity) assay.

2. Materials and methods

2.1. Drugs and chemicals

Human Recombinant Erythropoietin (rHuEPO) was purchased from Janssen-Cilag (Neuss, Germany). Ascorbic acid, 2,2′-azo-bis-amidinopropane (ABAP), EDTA, Iron (III) chloride hexa-hydrate, KMBA (α-cheto-γ-(methylthiol)butyric acid), 3-morpholinosydnonimine *N*-ethylcarbamide (SIN-1) and sodium chloride physiological solution were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Animals

Male Wistar healthy rats (n = 36; body weight 200–250 g) were purchased from Harlan, Italy. The animals were fed standard laboratory chow and tap water ad libitum and were not employed for at least one week after their delivery to the laboratory. They were housed in metabolic cages, three in a cage, in temperature-controlled rooms on a 12 h light cycle at 22–24 °C and 50–60% humidity.

2.3. Experimental design

Male Wistar healthy rats were subjected to sub-acute treatment with rHuEPO, administered subcutaneously. Choosing a short-term treatment (7 total days, treatments on the 1st, 3rd and 5th day) with high doses of the drug (7.5, 15, 30 μg/kg) it was made to mimic the abuse of this drug in sport and to analyze the consequences of this condition on the oxidative stress.

Briefly, after the acclimation time, rats were then randomly divided into four groups: group I or control group, animals (n = 9) treated with a subcutaneous administration of physiological solution on day 1, 3, 5; group II, animals (n = 9) treated with a subcutaneous administration of 7.5 μg/kg dose of rHuEPO on day 1, 3, 5; group III, animals (n = 9) treated with a subcutaneous administration of 15 μg/kg dose of rHuEPO on day 1, 3, 5; group IV, animals (n = 9) treated with a subcutaneous administration of 30 μg/kg dose of rHuEPO on day 1, 3, 5.

On day 7, rats were euthanized by a lethal dose of chloral hydrate (Sigma-Aldrich, Milan-Italy) and blood was collected by cardiac puncture using an EDTA coated tubes (Vacuette, Radnor, PA, USA). Plasma was obtained by centrifugation at 2500 rpm for 10' at 4 °C, and appropriately divided into aliquots for the performance of the various analyses. Urine, instead, were collected daily.

All experimental protocols were approved by the Animal Care and Use Committee of the University of Pisa, and were in compliance with the national and European guidelines for handling and use of experimental animals.

2.4. Evaluation of hematocrit and plasma erythropoietin concentration

To evaluate the plasma erythropoietin concentration was used the Human EPO immunoassay kit, Quantikine IVD (R&D systems, MN-USA), an Elisa based on the double-antibody sandwich method. Microplate wells, pre-coated with a mouse monoclonal antibody specific for EPO, were incubated with samples and erythropoietin bound to the immobilized antibody on the plate. After removing excess specimen or standard, wells were incubated with a rabbit anti-EPO polyclonal

antibody conjugated to horseradish peroxidase. A chromogen was added to the wells and was oxidized by the enzyme reaction to form a blue colored complex. The reaction was stopped by the addition of acid, which turned the blue to yellow. The amount of color generated is directly proportional to the amount of conjugate bound to the EPO antibody complex, which, in turn, is directly proportional to the amount of EPO in the specimen or standard. Assay results are measured

spectrophotometrically at 450 nm using a microplate reader (Multiskan FC, ThermoScientific). Hematocrit levels were analyzed with an automated cell counter (ADVIA® 120 Hematology System, Siemens, Munich, Germany).

2.5. TOSC assay

The total oxidant scavenging capacity (TOSC) assay is an in vitro method developed by the working groups of Regoli and Winston [20,21] that can be applied to pure antioxidant solutions as well as complex biological samples like fluids and tissues [17,18]. Peroxyl radicals were generated by thermal homolysis of 20 mM ABAP at 35 °C in 100 mM potassium phosphate buffer, pH 7.4. Hydroxyl radicals were generated at 35 °C by the iron plus ascorbate-driven Fenton reaction (1.8 μM Fe3+, 3.6 μM EDTA, and 180 μM ascorbic acid in 100 mM potassium phosphate buffer, pH 7.4). Peroxynitrite was generated from the decomposition of SIN-1 in the presence of 0.2 mM KMBA, 100 mM potassium phosphate buffer, pH 7.4, and 0.1 mM DTPA (Diethylene Triamine Penta Acetic Acid), at 35 °C. The concentration of SIN-1 was varied to achieve an ethylene yield equivalent to the iron–ascorbate and ABAP systems. Reactions with 0.2 mM KMBA were carried out in 10 ml vials sealed with gas-tight Mininert® valves (Supelco, Bellefonte, PA) in a final volume of 1 ml.