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A N I N T E R N A T I O N A L J O U R N A L

The Evolving Role of Erythropoietin in Lung of Irradiated Rats

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ABSTRACT

The aim of the present study is to evaluate the possible curative effect of erythropoietin (EPO) on radiation-induced damage to the lung and its renin angiotensin system. EPO (200 U/100g) was i.p. injected to male rats one hour post 6 Gy whole body gamma irradiation. The animals were sacrificed after 14 days post irradiation. Irradiation induced significant drop of haematological values, bone marrow (BM) count, lung oxidative stress markers, glutathione (GSH) and superoxide dismutase(SOD) associated with significant elevation of malondialdehyde (MDA), advanced oxidation protein product (AOPP) and nitric oxide (NO) besides serum inflammatory markers, tumor necrosis factor alpha (TNF-α) and lactate dehydrogenase (LDH). Also serum and lung renin angiotensin system markers, sodium (Na) and potassium (K) were elevated whereas calcium (Ca) was decreased. EPO treatment post irradiation has significantly ameliorated blood parameters and BM count also lung oxidative stress markers were improved associated with decreased serum Na, TNF-α, and LDH levels. Lung K and Ca showed no change compared to irradiated group. The findings of the present study suggest that EPO might contribute to enhance recovery of the lungs from radiation–induced damage due to its erythropoietic, anti-oxidative and anti-inflammatory effects.

Key words: erythropoietin; radiation; inflammation; oxidative stress.

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INTRODUCTION

Human and animal cells permanently react with oxygen. As a consequence of these reactions highly reactive molecules including free radicals are formed which leads to oxidative stress in cells. Oxidative stress is considered to be imbalance between the production of reactive oxygen species (ROS) and the biological ability to remove reactive intermediates [1].

Acute effects of radiation include hematopoietic cell loss, immune suppression, and potential injury to other sites such as the lung, kidney and central nervous system [2]. Acute radiation syndrome involves pathophysiological processes of organ dysfunction where the gut and the lung play a pivotal role. Radiation-induced lung fibrosis is a limiting complication in the treatment of haematological disorders involved in total body exposure to radiation [3]. Inflammation is the predominant early finding within irradiated lungs. This is followed by a second wave of inflammatory response that takes place 1–4 weeks after exposure, with inflammatory cell recruitment in the lungs. After irradiation, central inflammation is caused by increased expression of pro-inflammatory cytokines such as TNF-α [4]. The enzyme i-nitric oxide synthase (i-NOS) is very well known to be a significant part of the fibrotic pathway [5] and oxidative stress depletes alveolar epithelial glutathione levels. Irradiation is known to stimulate renin angiotensin system (RAS) [6].

Erythropoietin (EPO) has been widely used for treatment of anemia in chronic kidney disease and cancer chemotherapy-associated anemia [7] and for reduction of allogenic blood transfusion in surgery patients. Researches in the last decade have shown that EPO and its receptor are expressed in tissues other than those concerned in erythropoiesis including the brain, the reproductive tract, the heart, the spleen, and the lung [8]. There is a growing body of evidence that EPO may have important therapeutic roles in preventing or ameliorating ischemic or toxic damage to critical organs through anti-inflammatory actions [9]. EPO may exert the anti-inflammatory actions either directly by antagonism of pro-inflammatory cytokines (such as TNF-α) or indirectly by mitigation of tissue injury [10].

Erythropoietin, and other haematopoeitic cytokines, use Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway that is used by various components of renin angiotensin system (RAS) which have been identified in the lung [11]. The present study aims to evaluate the outcome of EPO on hematopoietic reconstitution, lung oxidative stress and RAS in irradiated rats.

MATERIALS AND METHODS

Mature male albino rats of pure strain ranging from 110-150 g body weight; 8 weeks old were obtained from the Egyptian Holding Company for Biological Products and Vaccine (Cairo, Egypt). The animals were maintained under standard conditions of ventilation, temperature, light and humidity and allowed free access to standard pellet diet and tap water. All animal treatments were conducted according to the Ethics Committee of the National Research Centre and in accordance with the recommendations for the proper care and use of laboratory animals (HIN publication No. 85-23, revised 1985) in accordance with international ethical considerations.

Radiation facility

Whole body γ- irradiation was performed with a Canadian ^{137}Cs Gamma Cell-40 biological irradiator located at the National Centre for Radiation Research and Technology, Cairo, Egypt; at a dose rate of 0.49 Gy/min. Rats were exposed to

whole body γ- radiations delivered as an acute single dose of 6Gy to induce lung damage [12].

EPO treatment:
Erythropoietin was purchased as ampoules (Epoetin) from SEDICO, Egypt. Rats received a single i.p. injection at a dose of 200 U/100g [5] and were sacrificed after 14 days to detect radiation damage.

Groups of Animals
Animals used in the present experiments included 4 groups of 6 males categorized into: 1- Control untreated rats (C). 2- Group of rats injected i.p. with 200 U/100g of (EPO). 3- Group of rats exposed to 6Gy whole body gamma irradiation (R). 4- Group of rats exposed to 6Gy gamma irradiation and injected i.p. with a single dose of 200 U/100g EPO, one hour post irradiation (R +EPO). All animals groups were sacrificed after 14 days.

Samples collection
After 14 days, animals were sacrificed and blood was immediately collected by heart puncture. Part of the blood was placed on ethylene diamine tetra acetic acid (EDTA) from Sigma Aldrich Chemical Co. St Louis, MO, USA, for haematological analysis and the rest was separated by centrifugation and stored as serum at -20°C until analyzed.
Peripheral blood cell counts were analyzed in a MASCOT Multi-species Hematology System (CDC Technologies, Oxford, CT, USA).
Bone marrow suspension count: Femur bones were cleaned and chipped. The bone marrow was blow out into 5 ml saline solution, pooled and mixed by drawing and expelling it several times from the syringe without needle. Total number of bone marrow nucleated cells were counted using trypan blue on a haemocytometer [13].

Biochemical analysis
All chemicals and reagents were purchased of pure chemical materials from Sigma Aldrich, St Louis, MO, USA. Measurements in the lungs: For assessment of oxidative stress biomarkers a portion of the lung was weighed and 10% weight/volume (W/V) tissue homogenates were prepared in 0.1M phosphate buffer (pH 7.4) using Teflon homogenizer (Glas-Col, Terre Haute, Ind., USA). The homogenates were centrifuged at 10,000g for 15 min. Aliquots of supernatants were separated for use. Lung tissues malondialdehyde (MDA), an end product of lipid peroxidation, was determined according to Yoshioka et al. [14], and nitric oxide (NO) was estimated according to Cortas and Wakid [15], advanced oxidation protein product (AOPP) was determined according to Witko-Sarsat et al. [16], Superoxide dismutase (SOD) activity was assayed by the method of Minami and Yoshikawa [17], wehereas (GSH) was determined according to Beutler, et al. [18]. Sodium and potassium were determined according to Kim et al. [19], calcium was determined according to Janssen and Helbing [20] using Quimica Clinica Aplicada, S.A. Co., kits. Serum LDH concentration was determined according to Kachmar and Moss [21] and TNF-α concentrationwas assayed by commercially available ELISA kit (Quantikine, R & D Systems, Minneapolis, MN) and measured according to Aramachi [22]. All determinations were done using T60 UV/VIS spectrophotometer (PG Instruments, London, UK).

Statistical analysis:
Comparisons among groups (n= 6) were performed by computer program SPSS (Chicago, IL, USA) version 15. Statistical significance was determined using Student t-test. Differences between means were considered significant at $P < 0.05$. The values are expressed as means ± SD (standard deviation).

RESULTS

EPO injection (200 U/100g) resulted in elevated Hb, Hct and platelets and no change was observed in leucocytes and erythrocytes as well as viable bone marrow cells in non irradiated rats compared to the control 14 days after treatment. All blood parameters and BM cells investigated 14 days post irradiation showed significant ($p \leq 0.05$) decreases compared to the control animals. The decrease in RBC, WBC, Hb, Hct and platelets by irradiation was significantly elevated in rats treated with EPO (Figure 1) compared to irradiated group. Accelerated bone marrow reconstitution was also observed by EPO treatment to irradiated animals compared to irradiated group (Figure 2).

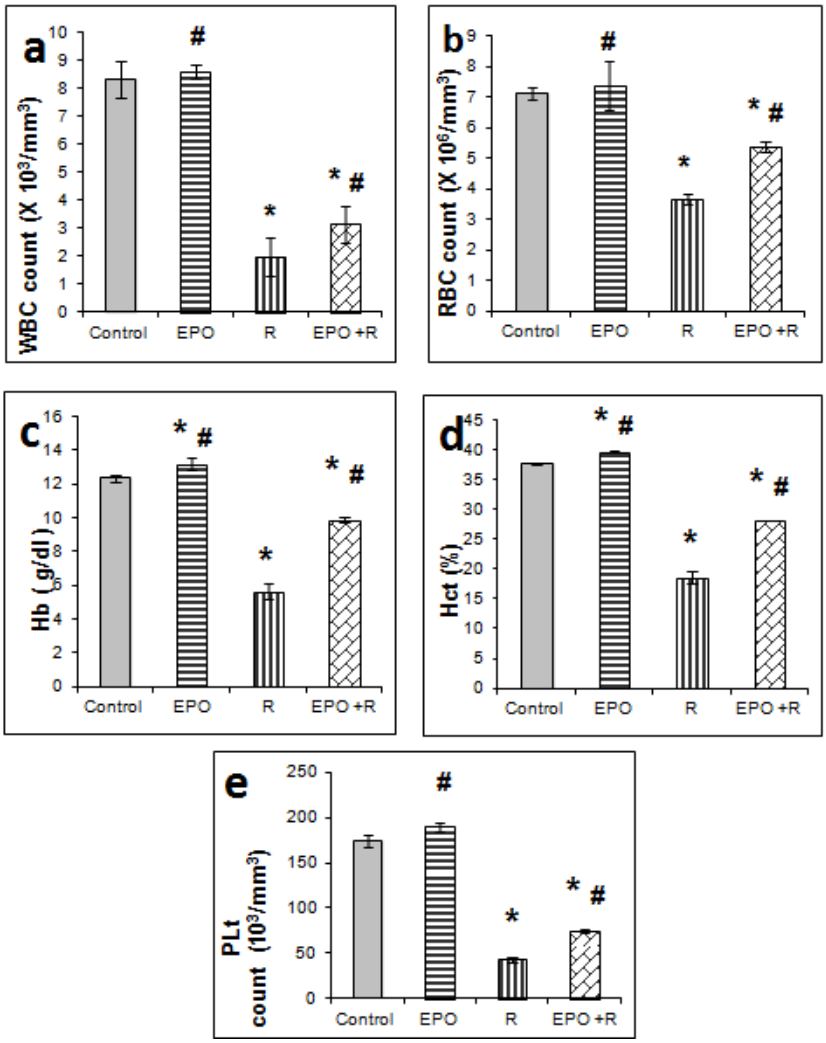

Figure 1: Effect of 6 Gy gamma irradiation and erythropoietin on blood parameters:
*: significantly different from that of the control.
#: significantly different from that of irradiated rats.

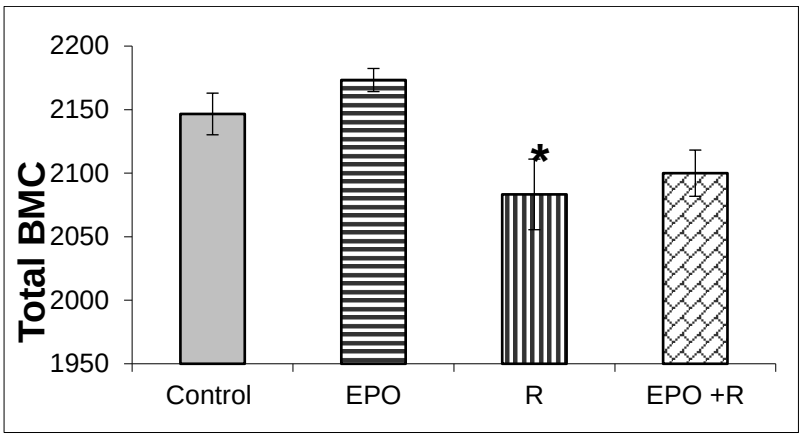

Figure 2: Effect of 6 Gy gamma irradiation and erythropoietin on total bone marrow count X 10^3.
*: significantly different from that of the control.
#: significantly different from that of irradiated rats.

Whole body exposure of rats to gamma radiation (6Gy) provoked oxidative stress demonstrated by significant ($p \leq 0.05$) increase of MDA, NO, AOPP levels of lung tissues associated with significant decrease of GSH content and SOD activity as compared to their respective values in the control group (Table 1) . Administration of EPO 1 hour post irradiation induced significant decrease ($p \leq 0.05$) of oxidative biomarkers levels of lung tissues and significant increases of antioxidants compared to the irradiated group.

Table 1. The effect of erythropoietin (EPO) on glutathione (GSH) content, malondialdehyde (MDA), nitric oxide (NO), advanced oxidation protein products (AOPP) levels and super oxide dismutase (SOD) activity in the lung of rats 14 days post 6Gy γ -irradiation (R).

	Parameter				
Groups	GSH (mg/ gm tissue)	MDA (nmole/ gm tissue)	NO (μmol / gm tissue)	AOPP (n mol /gm tissue)	SOD (u /gm tissue)
Control	18.03±0.4	112.5±11.1	65.6±6.8	250.2±15.02	367.4±11.9
EPO	17.31±1.1*#	110.5±13.2*#	64.3±5.5*#	254.5±15.36*#	366.1±14.9*#
R	9.5±1.5*	194.4±17.7*	91.6±8.6*	356.7±12.74*	243.2±10.5*
EPO +R	14.1±0.69*#	137.9±15.9*#	67.1±7.7*#	282±17.84*#	361.1±14.7*#

*: significantly different from that of the control.
#: significantly different from that of irradiated rats.

Table 2. The effect of erythropoietin (EPO) on TNFα, LDH, Na, K, and Ca in the serum of rats 14 days post 6 Gy γ -irradiation (R).

	Parameter				
Groups	TNFα (Pq/ml)	LDH (U/L)	Na (mmol /L)	K (mmol /L)	Ca (mg/dl)
Control	133.9±9.24	11.69±1.5	111.6±13.6	3.86±0.07	10.3±0.22
EPO	151.6±3.75#	9.42±0.49#	99.6 ±15.5*#	3.93±0.08#	9.26±0.13*#
R	198.6±7.96*	34.41±0.61*	168.8±18.4*	5.23±0.08*	8±0.03*
EPO +R	173.6±2.86*#	24.2±1.2*#	138.3±10.7*#	4.03±0.02#	8.7±0.2*#

*: significantly different from that of the control.
#: significantly different from that of irradiated rats

The present results (Table 2) demonstrated that EPO treatment decreased serum Na and Ca values compared to the control. A significant increase of TNFα and LDH values were induced by irradiation, whereas after 14 days from EPO treatment to irradiated rats, a significant decrease was detected in these values compared to the irradiated group. The irradiated animals showed significant elevations of Na and K accompanied by a significant decrease of Ca in both serum and lung (Tables 2 and 3). EPO treatment to irradiated animals significantly decreased serum Na and K and elevated Ca values compared to the irradiated group, whereas, no significant change was noted in lung K and Ca values.

Table 3: The effect of erythropoietin (EPO) on lung Na, K, and Ca of rats 14 days post 6 Gy γ -irradiation (R).

	Parameter		
Groups	Na (mmol / gm tissue)	K (mmol / gm tissue)	Ca (mg/ gm tissue)
Control	0.36±0.01	0.11±0.01	0.46±0.022
EPO	0.37±0.05#	0.13±0.03#	0.45±0.013#
R	0.42±0.02*	0.19±0.01*	0.42±0.03*
EPO +R	0.39±0.01*#	0.18±0.07*	0.43±0.02*

*: significantly different from that of the control.
#: significantly different from that of irradiated rats.

DISCUSSION

Exposure to ionizing radiation produces ROS (hydroxyl radicals, superoxide anion radicals and hydrogen peroxide) which causes antioxidant /oxidant imbalance and cause cellular damage [23]. Antioxidant/oxidant balance is necessary to maintain redox homeostasis especially during oxidative stress conditions [24].

Irradiation of the animals at 6 Gy resulted in a significant decrease in WBCs, RBCs, platelets, Hct value, Hb content and bone marrow viable cells. The hematological values decrease is attributed to a significant reduction of hematopoietic stem cells (HSC) and impairment of its self-renewal via activation of the specific cellular pathways by irradiation [25]. Nevertheless, the decrease in RBCs count and thus Hb content might also be attributed to increased permeability of cell membrane, leading to erythrocyte haemolysis [26]. The decrease in WBCs is the consequence of radiation-induced lipid peroxidation and damage of their cell membranes rich in polyunsaturated fatty acids which coincides with elevated MDA content. Irradiation induced leucopenia has likewise been reported in gamma irradiated mice as a direct concequence of lymphopenia and neutropenia following irradiation [27]. The radiation induced decrease in Hb content is attributed to the decline in the observed number of red blood cells and to decreased Hb affinity for oxygen [28]. Hct decrease can be attributed to the failure of erythropoiesis, destruction of mature cells, or increased plasma volume [29].

EPO treatment attenuated the decrease in blood parameters and BM cells in irradiated animals. This is attributed to the supportive effect of cytokines to bone marrow cells in reconstitution of haematopoietic organs [30] and to EPO role in treatment of anemia and stimulation of erythropoiesis [7]. Erythropoietin binds to EPO receptors on RBCs surface and activates JAK2 cascade [31]. This pathway can serve as a point of cross talk between the components of locally present RAS in the bone marrow and haematopoiesis.

The function of the pulmonary RAS seems to be of particular importance since it plays a role in the pathogenesis of lung diseases related to lung injury and fluid homeostasis [32]. Irradiation is known to stimulate RAS [6]. ANG II is a hormone that causes vasoconstriction and tubular reabsorption of sodium. It is a growth

promoting substance that is implicated in lung fibrosis [32]. The significant increase of Na and K values in serum and lung of irradiated animals besides the decrease of Ca in the present results is an indication of activation of RAS inducing salt retention [33]. It is also attributed to destruction of mature erythrocytes due to partial damage of the natural barriers for Na and K movements by gamma radiolysis [34] and the inhibitory effect of ionizing radiation on Ca ion channels [35], or radiation induced elevation of aldosterone which contributes to the loss of Ca [36]. In the present study treatment of irradiated rats with EPO exerted significant drop of Na and K and elevation of Ca in the serum and significant decrease of lung Na which could be attributed to EPO anti-apoptotic and anti-oxidative properties preventing damage to critical organs [37].

Antioxidant enzymes are considered to be the first line of cellular defense against oxidative damage [38]. The current study showed significant inhibition of GSH content and SOD in parallel to increment of MDA, NO, AOPP in γ-irradiated rats. GSH present in the lung lining plays a crucial role in protecting the lung from oxidative stress by detoxifying exogenous toxicants and quenching ROS [39]. Radiation-induced oxidative stress depletes alveolar epithelial GSH and SOD.

Inflammation is the predominant early histological and physiological finding within irradiated lungs. This is followed by a second wave of inflammatory response that takes place 1–4 weeks after exposure, with inflammatory cell recruitment in the lungs [40]. It is well known that NO modulates cell radiosensetivity and immunological response [41] and ionizing radiation increase NO production by inducing NOS expression and stimulating constitutive NOS [42]. i-NOS is very well known to be a significant part of the fibrotic pathway [5] which coincides with the present results. In the same way, the present increase of AOPP levels might be attributed to the interaction of proteins with ROS [43].

In the current study, oxidative stress parameters in the lungs were significantly improved by EPO post-administration. EPO is regarded as a general tissue-protective cytokine, a strong antioxidant and increases the activity of antioxidant enzymes, such as SOD, and has been reported to decrease MDA levels in hypoxic-ischemic organ injuries [9]. It is also shown that EPO has protective effects associated with acute lung injury model by inhibiting leukocyte accumulation and reducing oxidative stress-associated lipid peroxidation [44]. Hypoxia- induced EPO production regulated by hypoxia- inducible factor-1(HIF-1) has been observed in astrocytes in the brain and endothelial cells [45] suggesting that EPO may mediate a number of organ responses to low oxygen tension, beyond simple erythropoiesis.

Elevated TNFα in the present study is explained by [46] on the basis that immunologically mediated inflammation undoubtedly plays a central role in airway inflammation. In the same way LDH elevation, as an indication of tissue and cellular damage, was attributed to irradiation induced leakage of cytosolic enzymes such as LDH [47]. Both acute and chronic inflammation may be involved in radiation-induced late organ damage, as anti-inflammatory treatments have been demonstrated to be beneficial regarding late organ damage/dysfunction [48]. The improvement of TNFα and LDH exerted by EPO seemed to be related to its anti-apoptotic, anti-oxidative, and anti-inflammatory properties as well as its angiogenic effect [49]. EPO may also exert anti-inflammatory actions either directly by antagonism of pro-inflammatory cytokines such as TNFα or indirectly by mitigation of tissue injury [9].

CONCLUSION

Based on the results obtained in the current study, it appears that EPO contributes to attenuation of lung injury via its antioxidant and anti-inflammatory effects.

AUTHORS' CONTRIBUTIONS

All authors participated equally in this work.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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Erratum

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