Erythropoietin Protects Against Exertional Rhabdomyolysis-induced Acute Kidney Injury in Association with Preferential M2 Macrophage Polarization and Hemeoxygenase-1 Activation

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Abstract

Background: Exertional rhabdomyolysis (ER)-induced acute kidney injury (AKI) is a serious health threat associated with strenuous physical exercise. Erythropoietin (EPO) is a pleiotropic hormone with its immunomodulator function still unclear. We investigated the renoprotective effect of EPO in EXR-induced AKI, with an emphasis on macrophages phenotypic polarization and associated hemeoxygenase1 (HO1) bioactivity.

Methods: Strenuous exercise was applied to rats, either or not preceded by EPO alone or combined with the HO1 enzyme blocker, Zinc protoporphyrins (Zn-PP). Serum levels of creatine phosphokinase, myoglobin, urea nitrogen, creatinine, and carboxyhemoglobin (COHb) % were estimated. In addition, we examined the inflammatory cytokines IL10 and TNF α , macrophages phenotypic markers, HO1 expression, and renal pathology.

Results: EPO pre-treatment resulted in significant decreases in blood urea nitrogen, serum creatinine and myoglobin, tubular injury score, and intratubular cast % in addition to TNF-α decrease, IL10 increase with a preferential switching of macrophage polarization to the reparative M2 phenotype as anti-inflammatory effect. Furthermore, EPO pre-treatment was associated with an increase in HO1 protein expression and COHb%. Such effects were significantly reversed when HO1 activity blocker, Zn-PP, was co-administered.

Conclusion: Our findings highlight the value of EPO as a prophylactic therapeutic agent against EXR-induced AKI. Additionally, they suggest an underpinning role for HO1 in the EPO-induced modulation of macrophage polarization towards the M2 phenotype.

Keywords: Erythropoietin, M2 macrophage, Heamoxygenase-1, Exertional rhabdomyolysis-induced acute kidney injury

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1. Introduction

Exertional rhabdomyolysis (EXR) refers to damage and necrosis of skeletal muscle fibers after engaging in intense physical activity [1, 2]. The condition is common among athletes and military personnel [1, 3, 4]. Acute kidney injury (AKI) is considered one of the most leading complications of rhabdomyolysis. The prevalence of AKI ranges from 10-50% [5], and has generally been associated with increased renal morbidity and mortality [6].

The EXR-induced damage of skeletal muscle fibers occurs through direct damage of the sarcolemma and/or electrolyte disturbance. Strenuous exercise causes depletion of cell energy, which inhibits the Na^+/K^+-ATPase and Ca^{2+}-ATPase pumps. The resultant intracellular accumulation of Ca^{2+} and Na^+ induces cell damage through the activation of Ca^{2+}-dependent phospholipases and proteolytic enzymes or osmotic lysis, respectively. Subsequently, intracellular proteins including creatine phosphokinase and myoglobin leak into the circulation [1]. Myoglobin is a particular key factor in the pathogenesis of rhabdomyolysis-induced AKI. After glomerular passage, it is reabsorbed and catabolized by the renal tubular cells, which causes them to undergo oxidative stress, apoptosis and vasoconstrictive cytokine responses [5, 7].

Current studies propose rhabdomyolysis-induced AKI as an inflammatory disease, in which macrophages play an evitable role [8-11]. The kidney's resident macrophages constitute a principal element of the innate immunity and orchestrate both the initiation and resolution of inflammation [12, 13]. They are divided into classically activated macrophages (M1), which secrete pro-inflammatory cytokines and bactericidal reactive oxygen and nitrogen species, and alternatively activated macrophages (M2), which mediate resolution and repair of inflammation [14]. A growing body of evidence suggests that accelerated macrophages polarization towards the M2 phenotype is of great value for alleviation and repair of renal EXR-induced AKI [9-11].

Erythropoietin (EPO) is a glycoprotein hormone that is responsible for hemopoiesis, and has been traditionally used for the treatment of anemia. Besides hemopoiesis, recent studies have demonstrated a role for EPO in healing and repair through anti-apoptotic and anti-inflammatory effects [15, 16]. Furthermore, some studies have identified macrophages as one of the immune-targets for the anti-inflammatory effect of EPO, which inhibits their release of pro-inflammatory cytokines [17-19]. Despite this knowledge, the mechanistic basis for the macrophage-targeted anti-inflammatory effect of EPO and its therapeutic implications remain elusive.

Hemeoxygenase-1 (HO1) is a rate-limiting enzyme for the degradation of the heme protein into biliverdin, resulting in release of carbon monoxide (CO) and free iron [20]. In addition, HO1 and its CO byproduct have been previously reported to restore homeostasis in acute and chronic inflammation [21-23], or to promote macrophage switching to the M2 phenotype [22, 24].

In the present study, we investigated the potential protective effects of EPO pre-treatment against EXR-induced AKI, with a particular emphasize on the anti-inflammatory role of macrophages. We further looked into the possible involvement of HO1 in macrophage switching to the anti-inflammatory M2 phenotype.

2. Materials and Methods

2.1 Experimental animals

Adult male albino rats weighting 200-220 gm were used in the experiment. Animals were purchased from Animal House, Faculty of Veterinary Medicine, Benha University (Egypt), and were housed in cages (three per cage) and maintained on prevailing atmospheric conditions

and room temperature. Food and water were provided ad libitum. Rats were subjected to an adaptation period of two weeks before experiments. This study was carried out in strict respect to the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was revised and approved by the Committee on the Ethics of Animal Experiments of the Faculty of Medicine, Benha University, Egypt.

2.2 Drugs and chemicals

Recombinant human EPO, was purchased from SEDICO Pharmaceutical (Cairo, Egypt). Zinc Protoporphyrin IX (Zn PP), specific inhibitor for HO1, was supplied by Sigma-Aldrich (MO, USA), in the form of powder and dissolved in sodium hydroxide, and then diluted to 1 mg/ml in normal saline solution.

2.3 Experimental groups and design

Rats were randomly divided into the following five groups (n= 6): (i) Control group, in which rats undergone warming-up exercise. (ii) EXR-induced AKI group, in which rats undergone exhaustive exercise training following warming up exercise training. (iii) EPO group, which received EPO at dose of 2000 I.U/ kg, I.P [25], ½ h before application of exercise. (iv) Zn PP group, which received Zn PP at a dose of 10 mg/kg, I.P [9], 24h before application of exercise. (v) Zn PP+ EPO group, which received 10mg/kg of Zn PP and 2000 I.U/ kg of EPO, I.P, 24h, and 1/2h, respectively, before application of exercise.

Exercise was executed by Rat treadmill (Panlab/Letica LE8706, Barcelona, Spain). The physiological warming-up exercise was undergone by all experimental groups, and was applied by horizontal running at speed of 17cm/sec for 15 min. Exhaustive exercise was applied by the last four groups, following the warming-up exercise, by running at gradient 10% for 10 min at speed of 30cm/sec and then 37cm/sec. This followed by running at 44cm/sec for 15 min and lastly the speed was fixed on 50cm/sec until rats became exhausted. To maintain steady running and ensure same exercise intensity, a shock grid (0.97 mA, 3 Hz), placed at the back of the treadmill, was used to discourage the rats from stopping while the treadmill belt was moving [25]. The rats ran until exhaustion, which is defined as remaining on the shocker plate for more than 10‒15 seconds [26], and time to exhaustion was recorded.

Recovery period of 24h was allowed for all groups, and then rats were sacrificed under general anesthesia using intra-peritoneal injection of pentobarbital sodium (40mg/kg). Blood samples were collected; a part in a heparinized syringe then placed on ice for carboxyhemoglobin assessment while, the rest in dry clean test tube then left at room temperature to clot. Serum was separated by centrifugation at 3000 rpm for 15 min and was stored at $-80\,^{\circ}$ C for other biochemical analysis.

Prior to the renal dissection, they were perfused with cold phosphate buffered saline (PBS) solution, pH 7.4, containing 0.16 mg/ml heparin to remove any red blood cells and clots. One kidney was homogenized using a Mixer Mill MM400 (Retsch, Germany) in 5-10 ml of phosphate buffer per gram tissue then centrifugation for 15 minutes at 4° C was performed. The supernatant was removed for biochemical assay. Half of the other kidney was used for real time PCR analysis while, the other half was fixed in 10% neutral buffered formalin and prepared for histopathology and immunohistochemical analysis.

2.4 Measurement of biochemical indices related to EXR and AKI

Measurement of serum creatine phosphokinase (CPK) was performed using the Creatine Kinase Activity Assay Kits (Sigma, MO, USA). The principal of the assay relies on coupled enzyme reaction [27]. Measurement of blood urea nitrogen (BUN) and creatinine levels was performed by enzymatic colorimetric method, using commercially available kits (Diamond Diagnostics Company, Cairo, Egypt). Serum myoglobin was measured using a commercial ELISA kit (Abcam, MA, USA) on diluted samples (1:5) according to the manufacturer's instructions. A standard curve was drawn, and a four-parameter algorithm equation was used to calculate the myoglobin concentrations in each sample.

2.5 Cytokine and Carboxyhemoglobin (COHb) % Measurement

Renal levels of TNF α and IL10 were assessed by specific rat ELISA kits (Abcam, Cambridge, UK), according to the manufacturer's instructions. COHb % levels were assessed by spectrophotometer calibrated for rat serum (Jenway, Bibby Scientific Ltd., Staffordshire, UK) [28]. Most of the endogenously released CO is stems from heme degradation, which is a reliable indicator for heme metabolism and the related HO1 activity [29].

2.6 RNA extraction and real-time PCR analysis for M1- and M2- exclusive genes

Based on the exclusive pattern of expression of the M1 and M2 subtypes of macrophages, we utilized Early growth response protein 2 (Egr2) as M2-marker, whereas Cluster of Differentiation 38 (CD38) as M1-marker [30, 31]. Total RNA was isolated from 25mg tissue using total RNA purification kit (Jena Bioscience Germany). The concentration and purity of the RNA were determined by measuring the absorbance at 260 nm and 280 nm by UV spectrophotometer (Optima SP-3000, Japan). Pure RNA has an A260/A280 ratio of 1.9–2.3.

RNA (5 μg) was then reversed transcribed using revert aid TM first strand cDNA synthesis kit (Ferments life science, USA) then cDNA was subsequently amplified with the Syber Green PCR Master Kit (Applied Biosystems, USA)

One-step RT-PCR using QuantiTectVR SYBRVR Green RT-PCR master mix kit Cat No./ID: 204243 (Qiagen, GmbH) supplied by clinilab (Egypt), was done in ABI Prism 7900HT (Applied Biosystem, Foster City, CA), QuantiTect SYBR Green RT-PCR Kits contain an optimised, ready-to-use master mix using SYBR Green I. QuantiTect SYBR Green RT-PCR master mix also contains dUTP, enabling pre-treatment with uracil-N-glycosylase (UNG) prior to starting PCR, which ensures that any contaminating PCR products do not affect subsequent PCR reactions, the prepared reaction components were done in 96 well PCR plate using Real-time cycler conditions of 50 oC, 30 min, (Reverse transcription) 95 oC, 15 min, (Initial denaturation) followed by 40 cycles of 94 oC, 30 s, 55 oC, 1 min and 72 oC, 1 min for Denaturation, Annealing, Extension steps respectively. The housekeeping Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as a reference gene.

Data analysis: According to the RQ manager program ABI prism 7500 the Boxplot software, the data were produced as sigmoid-shaped amplification plots in which the number of cycles is plotted against fluorescence (when using a linear scale). The threshold cycle (CT) served as a tool for calculation of the starting template amount in each sample. Because the relative quantities of the assessed genes are normalised against the relative quantities of the endogenous control GAPDH gene, fold expression changes are calculated using the equation $2^{-\Delta\Delta ct}$ [32].

Rats' primer sequences of both forward and reverse (Operon, Inc., Huntsville, Alabama, Germany) were as follow:

Egr2 forward, CGCCACACCAAGATCCACC, reverse, AGCCCCAGGACCAGAGG, CD38 forward, TGCAACAAGATTCTTCTTTGGAGCA, reverse, CTCAGGATTTTTCACACACTGAAG, and GAPDH forward, ATGTGTCCGTCGTGGATCTGAC, reverse, AGACAACCTGGTCCTCAGTGTAG.

2.7 Histopathological examination of renal tissue

Longitudinal sections of the kidney were fixed immediately in 10% formalin, embedded in paraffin, sectioned at 4 μm thickness and were stained with hematoxylin and eosin (H&E). Tubule injury was defined by necrotic lysis, tubule dilation, cast formation or sloughing of cellular debris into the tubule lumen and was scored semi-quantitatively as previously described [33]. The scoring system was as follows; 0= no change in the tubules; 1= <25% of tubular injury (mild); 2= 25% to 50% of tubular involvement (moderate); 3= 50% to 75% of tubules showing characteristic change (severe) and 4= more than 75% of tubular damage (very severe). Five random fields for each kidney slide were examined, and the average score served as the tubular injury score of the kidney tissue sample. To assess intra-tubular cast formation a semi-quantitative score was calculated in five fields where 0= no casts were found in renal tubules; 1= 25% of tubules had casts; 2= 50% of tubules had casts; 3= 75% of tubules contained casts; and 4= all tubules contained casts [34]. The tissue sections were observed by a blinded

pathologist under Olympus microscope system (Olympus, Tokyo, Japan).

2.8 Immunohistochemical staining of HO1

Briefly, kidney sections were de-waxed and rehydrated in decreasing concentrations ethanol. After being treated with protein-blocking solution, for endogenous Peroxidase, in 3% H2O2 in methanol for 20 min at room temperature, the slides were incubated with rabbit polyclonal anti-murine HO1 antibodies (Cayman chemicals, MI, USA) (dilution 1:200) at 4 ° C overnight. Then, the sections were incubated with biotinylated secondary antibody (dilution 1:500) for 30 min at 37° C. This was followed by reaction with diaminobenzidine as chromogen and counterstaining with hematoxylin. Images were observed using an Olympus microscope system (Olympus, Tokyo, Japan).The HO1 positive immune-reactive cells were quantified by counting in 10 images of high-power magnification $\times$ 400 per section for each group using Image-Pro Plus program version 6.0 (Media Cybernetics Inc., Bethesda, Maryland, USA) in the Pathology Department, Faculty of Medicine, Benha University, Egypt. The data were expressed as the percentage of HO1 positive cells in the total examined area as observed by a pathologist in a blinded manner on coded slides.

3. Statistical analysis

All data were analyzed using the program Statistical Package for Social Sciences (SPSS) version 19 (SPSS Inc., Chicago, Illinois, USA). Data are presented as the mean $\pm$ standard deviation (SD). Comparisons of all parameters among the study groups were performed by using one-way analysis of variance (ANOVA) with LSD post-hoc to compensate for multiple comparisons. P value < 0.05 was considered statistically significant.

4. Results

Table 1: Physical, biochemical, inflammatory cytokines parameters, and COHb % in experimental groups

	Control group	EXR-induced AKI group	EPO group	Zn PP group	Zn PP+ EPO group
Time to exhaustion (min)	—	178±16	256±32[#]	103±19[#]	185±34[$]
CPK (U/L)	134±25	5644±234[*]	5551±303[*]	6912±409[*]	5605±156[*]
Myoglobin (ng/ml)	22±5	278±64[*]	166±41[*]	306±72[*]	266±34[*]
BUN(mg/dl)	9.5±1.1	38.1±1.3[*]	11.1±1.1[#]	43±1.9[*#]	37±1.8[*$]
sCr (mg/dl)	0.72±0.19	4.11±0.4[*]	0.98±0.11[*#]	5.38±0.7[*#]	3.7±0.67[*$]
TNF α (pg/ml)	7.35±0.94	41.3±2.9[*]	12.28±3.3[*#]	56.65±5.9[*#]	39.55±2.8[*$]
IL 10 (pg/ml)	276 ±30	121±9	773±58[*#]	103±5[*#]	385±25[$]
COHb (%)	0.26±0.04	0.55±0.04[*]	0.91±0.04[*#]	0.21±0.06[*#]	0.23±0.03[$]

Data are expressed as mean ± standard deviation SD; (n=6); *p< 0.05 significant difference compared with the control group while #p< 0.05 significant difference compared with the EXR-induced AKI group and $P< 0.05 significant difference compared with the EPO group. The carboxyhemoglobin expressed by log transformation for COHb. EXR-induced AKI; exertional rhabdomyolysis-induced acute kidney injury, EPO; erythropoietin, Zn PP; Zinc protoporphyrins, CPK; Creatine phosphokinase, BUN; blood urea nitrogen, sCr; serum creatinine, TNF α; tumor necrosis factor α, IL10; interleukins 10, COHb; carboxyhemoglobin.
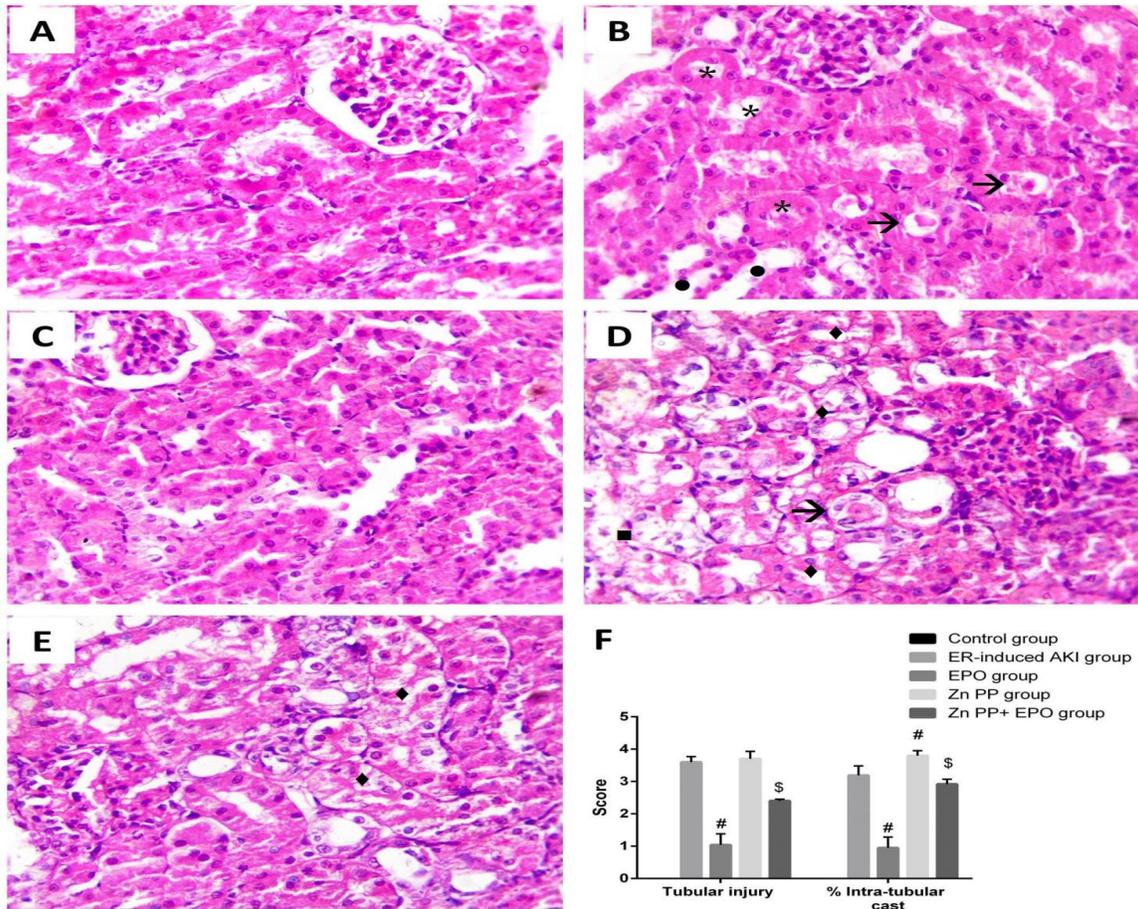
Figure 1: Histopathological picture of H&E-stained sections and scores of renal damage

(A); Normal kidney architecture of the control group. (B) EXR-induced AKI showing extensive cloudy swelling of tubular epithelial cells (*), tubular dilatation (●), and cast formation (→). (C) A nearly normal architecture with EPO group. (D) Marked renal damage with hydropic degeneration (♦), cast formation (→), and necrosis (■) as induced by pre-treatment with Zn PP alone in Zn PP group. (E) Tubular hydropic degenerative changes (♦) in the ZN PP + EPO group. (F) Tubular injury score and intra-tubular cast formation % for all experimental groups; data expressed as mean ± standard deviation SD; (n=6); #P< 0.05 significant difference compared with EXR-induced AKI group and $P< 0.05 significant difference compared with EPO group. Magnification= x400. EXR-induced AKI; exertional rhabdomyolysis induced acute kidney injury, EPO; erythropoietin, and Zn PP; Zinc protoporphyrins.
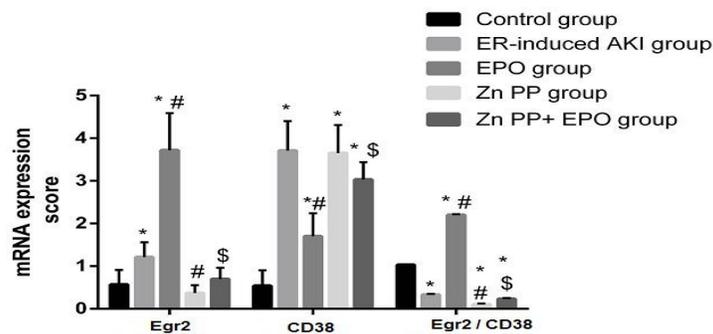

Figure 2: Effect of EPO pre-treatment on Egr2 and CD38 mRNA expression and their ratio

Data expressed as mean ± standard deviation SD; (n=6).[*]P< 0.05 significant difference compared with the control group, [#]P< 0.05 significant difference compared with EXR-induced AKI group and [$]P< 0.05 significant difference compared with EPO-treated group. EXR-induced AKI; exertional rhabdomyolysis induced acute kidney injury, EPO; erythropoietin, Zn PP; Zinc protoporphyrins, Egr2; Early growth response protein 2, CD38; Cluster of differentiation 38
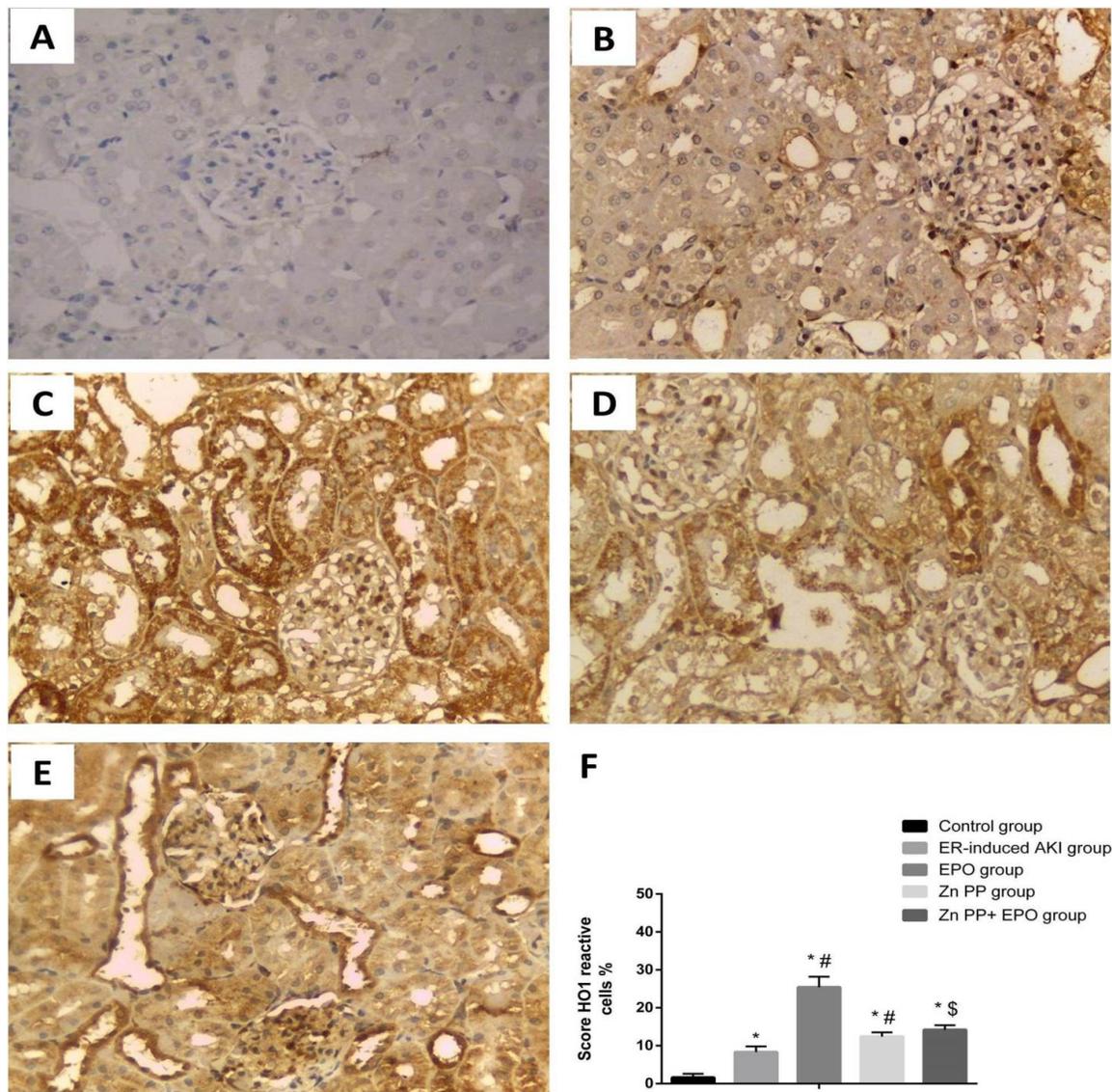

Figure 3: Immunohistochemical staining of renal tissue for HO1 expression and % of immunoreactive cells score

(A); Control group, (B); EXR-induced AKI group, (C); EPO group, (D); Zn PP group, (E); Zn PP + EPO group and (F); % of immunoreactive cells score; data expressed as mean ± standard deviation SD; (n=6); [*]P< 0.05 significant difference compared with the control group, [#]P< 0.05 significant difference compared with EXR-induced AKI group and [$]P< 0.05 significant difference compared with EPO group. Magnification= x400. EXR-induced AKI; exertional rhabdomyolysis induced acute kidney injury, EPO; erythropoietin, and Zn PP; Zinc protoporphyrins.

4.1 Effect of EPO pre-treatment on Time to exhaustion (Table 1)

All experimental rats appeared physically sound, dynamic, and alert before the application of before the application of exhaustive exercise to induce ER and the subsequent AKI. After the intensive exercise was applied to the EXR-induced AKI group, rats showed signs of fatigue and suffered a stasis accompanied by a shorter time to exhaustion compared to the control group ($P < 0.05$). Zn PP group showed the same signs of exhaustion, in addition to sub-conjunctival hemorrhage, and early exhausted than other groups with the significant least time to exhaustion ($P < 0.05$). Conversely, EPO group tolerated better to the exhaustive exercise, showed higher tolerance; scoring the longest time to exhaustion among the groups ($P < 0.05$). On the other hand, when Zn PP preceded EPO administration, such effects were reversed and Time to exhaustion was significantly decreased versus EPO group ($P < 0.05$).

4.2 Effect of EPO pre-treatment on renal function indices (Table 1)

Compared to the control group, serum myoglobin and CPK levels were significantly increased in EXR-induced AKI, EPO, Zn PP and Zn PP+EPO groups ($P < 0.05$). The differences of CPK levels among these groups were statistically non-significant, except for the Zn PP group that showed significantly higher levels compared to the EPO group ($P < 0.05$).

Concerning serum levels of BUN and Cr, they were significantly elevated in the EXR-induced AKI group as compared to the control one ($P < 0.05$). Moreover, Zn PP group exhibited significantly higher levels of BUN and Cr than those in other groups ($P < 0.05$). On the contrary, EPO group exhibited significant decreases in serum BUN and Cr levels when compared to EXR-induced AKI group. Meanwhile, when Zn PP preceded EPO administration, it significantly reversed EPO effect ($P < 0.05$).

4.3 Effect of EPO pre-treatment on renal architecture (Figure 1)

The biochemical alterations were simultaneously confirmed by changes in the renal tissue architecture. Compared to the normal renal structure shown for the control group (Fig 1A), histopathological examination of H&E-stained tissue sections form the EXR-induced AKI group showed parenchymal changes including cloudy swelling of tubular epithelial cells, tubular dilatation and some cast formation (Fig 1B). These histopathological lesions were largely ameliorated in the EPO group, which showed a nearly normal renal architecture (Fig 1C). Tubular injury score and intratubular cast % were significantly decreased versus EXR-induced AKI group ($P < 0.05$) (Fig 1F). On the contrary, when Zn PP preceded EPO administration group, it diminished the effect of EPO, as evident by lesions of hydropic changes, necrosis, and cast formation, as well as the significant increase in tubular injury score and intra-tubular cast % versus EPO group ($P < 0.05$) (Fig. 1E and 1F). Furthermore, Zn PP group showed marked renal damage and demonstrated comparable tubular injury score and intra-tubular cast % to the EXR-induced AKI group ($P < 0.05$) (Fig. 1D and 1F).

4.4 Effect of EPO pre-treatment on Egr2 and CD38 mRNA expressions (Figure 2)

It was observed that Egr2 mRNA expression was significantly upregulated in EPO group compared to the EXR-induced AKI group ($P < 0.05$). This effect was significantly attenuated when EPO was preceded by Zn PP ($P < 0.05$). Concerning renal CD38 mRNA expression, it was significantly upregulated in EXR-induced AKI group compared to the control group ($P < 0.05$). Moreover, the EPO group showed significant downregulation in its expression when compared to EXR-induced AKI group ($P < 0.05$). However, Zn PP abolished the effect of EPO and caused significant upregulation in renal CD38 mRNA expression compared to EXR-induced AKI group ($P < 0.05$).

Afterwards, we calculated the M2 (Egr2) / M1 (CD38) ratio. The control group showed a balance between M1 and M2 macrophages expression. A decreased M2/M1 ratio was observed in EXR-induced AKI group, while it was reversed with EPO pre-treatment. On the other hand, blocking HO1 activity in Zn PP and Zn PP+ EPO groups resulted in a significant decrease in M2/M1 ratio when compared to the EPO group ($P < 0.05$).

4.5 Effect of EPO pre-treatment on inflammatory cytokines (Table 1)

Levels of TNF α were significantly higher in the EXR-induced AKI group compared to the control (P < 0.05). Furthermore, this cytokine showed significantly downregulated in the EPO group compared to EXR-induced AKI group (P < 0.05). However, such effect was significantly reversed when EPO was preceded by Zn PP administration (P < 0.05). Renal TNF α in Zn PP group was significantly higher than those in other groups (P < 0.05).

Renal levels of the anti-inflammatory cytokine IL10 were significantly higher in the EPO group compared to EXR-induced AKI group (P < 0.05). By contrast, Zn PP intervention in Zn PP and Zn PP+ EPO groups resulted in significant decrease of the cytokine production compared with the EPO group (P< 0.05). Moreover, it was the least in Zn PP group than in other groups (P < 0.05).

4.6 Effect of EPO pre-treatment on HO1 expression (Figure 3) and COHb% (Table 1)

Immunohistochemical analysis of renal expression of HO1 protein showed faint or no immunoreactive cells in renal tubules in the control group (Fig 3A). In EXR-induced AKI group some renal tubules show considerable significant staining (Fig 3B) and was associated with an increase in COHb %, an indicator of heme degradation and endogenous CO release by HO1 activity when compared to the control group (Table 1). EPO group (Fig. 3C) showed highest HO1 expression and COHb % with respect to other groups (P < 0.05). Surprisingly, we observed that Zn PP intervention in Zn PP and Zn PP+ EPO groups (Fig 3D and 3E) showed significant much renal tubules staining (P < 0.05) as compared to EXR-induced AKI and control groups (Fig 3F) but with decreased COHb %. Moreover we observed in Zn PP group, COHb % was significantly the least among those in other groups (P < 0.05).

5. Discussion

AKI is one of the leading complications of ER and can cause serious renal complications. In the present study, we demonstrated the protective effect of EPO pre-treatment against the condition in a rat model of ER. In our model, rats exposed to exhaustive exercise training exhibited significant increase in serum CPK, a sensitive degradation product of myocytes that confirms the occurrence of ER. Acute strenuous exercise causes damage to skeletal muscles fibers either through direct damage of the cell membrane or hindrance of energy-mediated electrolyte pumps. Following muscular injury, subsequent release of tremendous amount of the intracellular CPK and myoglobin occurs [1]. The latter caused a deterioration of renal functions, as indicated by the significant increase in BUN and sCr levels [35], and extensive tubular damage, dilatation and cast formation as evident by histological analysis. These findings are in agreement with those previously described by Lin et al. [25], and further confirm the validity of their model to study EXR-induced AKI.

With regard to macrophage function, oxidative damage, ischemia and tubular cell death, serve as an important inducer of recruitment of innate immune cells. The outsets of AKI are typically followed by a rapid and abundant renal influx of blood monocytes that subsequently differentiate into macrophages [36, 37]. The AKI-associated innate cytokine responses direct these resident macrophages to switch to the pro-inflammatory M1 phenotype [38]. The latter is known to differentially express CD38 [30], as well as high levels of the pro-inflammatory cytokine TNF α [13]. Indeed, both markers were differentially expressed in the EXR-induced AKI group compared to the control, indicating a role for M1 macrophages in the development of the disease.

Pretreatment with EPO has improved renal function and ameliorated the inflammatory responses associated with EXR-induced AKI. This is consistent with a vast body of studies that described the pleiotropic effects of EPO for improved survival of renal tubular cells, increased renal and glomerular blood flow, improved endothelial regeneration and function, and reduced inflammation [39]. Although these protective effects can plausibly be linked to the interaction of EPO with local receptors, which are abundantly expressed on renal tubular cells and endothelial cells [40], our study rather focused on the potential contributing role of macrophages in protection against AKI. Previous studies have proposed macrophages as a new target in rhabdomyolysis

induced AKI [9, 26, 41]. As compared to the EXR-induced AKI group, the EPO group showed significant increase in renal IL10 and Egr2 mRNA expression, indicative of M2 switching [30]. Additionally, by calculating Egr2/CD38 ratio, we found M2 dominance in the contrary of M1 dominance in EXR-induced AKI.

This EPO-enhancing effect on M2 polarization in rhabdomyolysis induced AKI in rats is consistent with the findings of Wang et al. [42]. Moreover, Rubio-Navarro and his colleagues have studied the macrophage phenotypes associated with rhabdomyolysis induced AKI and demonstrated that myoglobin promotes an early inflammatory M1 response and differentiation towards M2 with M2 dominance but at day 7 after rhabdomyolysis induction [41]. Herein the M2 dominance occurred earlier with EPO preconditioning; 24h after rhabdomyolysis induction, which reflects an EPO-mediated acceleration of macrophages switching from M1 to M2 phenotype.

The EPO regulation of the polarization dynamics of renal macrophages is traced back to inhibition of NF-κB P65 subunit activation and subsequent activation of target gene expression [17] under inflammatory conditions [43, 44]. However, although our study of EXR-induced AKI does not tackle the molecular basis for the favorable EPO-induced M2 macrophage polarization, we explored the possible association of HO1 activity which might mediate macrophage polarization towards the M2 phenotype [24, 43]. In our study, EPO-treatment was associated with higher HO1 expression and activity. On the contrary, co-administration Zn PP as an HO1-enzyme inhibitor has significantly lowered HO1 activity and weakened M2 switching capacity compared to the EPO group. In addition, the effect of EPO on renal architecture and cytokine responses was similarly reversed under the inhibitor's effect. These findings support the notion that HO1 activity might mediate the renoprotective effect of EPO in AKI.

In this regard, previous studies demonstrated the stimulating effect of EPO on HO1 expression in hepatocytes after ischemia/reperfusion injury [45], neurons [46], astrocytes [47] and in monocyte of patient under hemodialysis [48]. Moreover, the stimulatory effect of EPO on renal HO1 expression has been described in vivo and in vitro [49], in addition to the stimulatory effect of HO1 on differentiation of M2 macrophages in models of protection against obesity-induced inflammation [50], diabetic cardiomypathy [51], diabetic gastroparesis [52], colonic inflammation [53] and atherosclerosis [54]. To our knowledge, our study is the first to suggest the same effect of EPO on HO1 expression in the kidney, unraveling an important physiological mechanism with therapeutic potential.

Surprisingly, we have noticed a non-significant change of myoglobin and CPK levels in EPO group, leading us to suggest that EPO has no direct protective effect on muscles, and the improved general health of EPO group was due to renal protective outcome. Conflicting results regarding identification and functionality of the EPO receptors in muscular tissue and across species were reported [55, 56]. We have also noticed that with respect to all other groups, Zn PP group exhibited severe renal damage; the least time to exhaustion, the highest levels of BUN, serum Cr, tubular injury score and intra-tubular cast % in addition to TNF α and CD38 mRNA expression, as well as a decrease in COHb %. Taken together, these findings suggest that the Zn PP blockage of HO1 activity aggravated renal injury and inflammation and have shed light on the crucial role of HO1 in EPO-conferred protection against EXR-induced AKI.

Unpredictably, the Zn PP group exhibited significant increase in renal HO1 protein expression, although it was associated with the significant decrease in COHb % versus control and EXR-induced AKI groups. This dichotomy could be explained by a negative feedback circuit that inhibiting the HO1 activity leads to subsequent compensatory activation of HO1 expression, although the molecular mechanisms underlying this effect remain unclear.

Finally, renal HO1 protein expression and COHb % were significantly increased in EXR-induced AKI group versus the control. The increasing HO1 expression in the EXR-induced AKI group gives rise to the importance of heme as a physiological inducer for HO1 expression and activity. This goes hands with the findings of Nath and his team, who initially reported HO1 expression as a rapid and protective reaction in rhabdomyolysis [57]. However this shock-response appears to be insufficient to protect the kidneys, due to associated

renal dysfunctions, being the pool of free heme is radically increased following rhabdomyolysis caused by dissociation from myoglobin and release of heme containing proteins and enzymes as cytochrome from damaged tubular cells [41].

6. Conclusion

To our knowledge, this study is the first to demonstrate the renoprotective effects against EXR-induced AKI by enhancing macrophages switching towards the anti-inflammatory M2 phenotype. In addition, the study provides an early evidence for the possible involvement the HO1 activation in the preferential M2 macrophage polarization under EPO treatment, which requires further molecular investigation.

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