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Erythropoietin attenuates LPS-induced microvascular damage in a murine model of septic acute kidney injury

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ABSTRACT

Acute kidney injury (AKI) is a frequent complication of sepsis, with a high mortality. Hallmarks of septic-AKI include inflammation, endothelial injury, and tissue hypoxia. Therefore, it would be of interest to develop therapeutic approaches for improving the microvascular damage in septic-AKI. Erythropoietin (EPO) is a well-known cytoprotective multifunctional hormone. Thus, the aim of this study was to evaluate the protective effects of EPO on microvascular injury in a murine model of endotoxemic AKI.

Male Balb/c mice were divided into four groups: control, LPS (8 mg/kg, ip.), EPO (3000 IU / kg, sc.) and LPS + EPO. A time course study (0–48 h) was designed. Experiments include, among others, immunohistochemistry and Western blottings of hypoxia-inducible transcription factor (HIF-1α), erythropoietin receptor (EPO-R), vascular endothelial growth factor system (VEGF/VEGFR-2), platelet and endothelial adhesion molecule-1 (PeCAM-1), inducible nitric oxide synthase (iNOS) and phosphorylated nuclear factor kappa B p65 (NF-κB).

Data showed that EPO attenuates renal microvascular damage during septic-AKI progression through a) the decrease of HIF-1 alpha, iNOS, and NF-κB and b) the enhancement of EPO-R, PeCAM-1, VEGF, and VEGFR-2 expression.

In summary, EPO renoprotection involves the attenuation of septic-induced renal hypoxia and inflammation as well as ameliorates the endotoxemic microvascular injury.

1. Introduction

Sepsis is a systemic inflammatory response elicited by an infection and has long been documented as a most common contributing factor for the development of acute kidney injury (AKI). Sepsis-associated AKI occurs in about 40% of patients with severe sepsis and septic shock [1]. There is strong evidence that it is related to a high mortality rate. Hitherto, no particular effective therapy has been developed to alter its natural history [2].

The development of innovative therapies for septic-AKI is critically dependent on an understanding of the basic mechanisms of the disease. However, the current knowledge of the pathophysiology mechanisms involved in AKI mediated by sepsis is still incomplete [3].

In addition to the typical tubular necrosis, several drivers for injury are currently recognized, as crucial players in septic related-AKI. They include inflammation, hypoxic stress, ischemia-reperfusion injury, tubular and /or mesenchymal apoptosis, cytokine-driven direct tubular damage and microcirculatory dysfunction [4].

Endothelial functions affected by sepsis include barrier function, hemostasis inflammation, and vascular regulation. Furthermore, numerous pathophysiological mechanisms have been proposed for microvascular dysfunction in sepsis-induced AKI: a) Endothelial injury, b) Microcirculatory hypo perfusion resulting in areas of tissue hypoxia, c) Excessive generation of nitric oxide (NO) by the inducible nitric oxide synthase (iNOS), d) peritubular capillary dysfunction and e) tubular damage induced by oxidative stress [5].

Novel therapeutic options for AKI protection should include the use of drugs with multiple favourable effects. In this regard, EPO has emerged as a multifunctional tissue-protective cytokine, which exerts anti-inflammatory, anti-oxidant, anti-apoptotic and pro-angiogenic effects in several tissues [6]. Moreover, it is known that rhEPO has been used for diminishing renal damage in several kidney injuries [7,8].

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The effect of EPO on prevention and treatment of AKI in patients is still very controversial. EPO administration minimized kidney lesions and the systemic inflammatory response, thereby decreasing AKI incidence in patients undergoing coronary artery bypass grafting [9,10]. However, EPO was inefficient in preventing AKI, reducing ICU stays and decreasing mortality in cardiac surgery patients [11–13].

Hence, the controversial outcomes in clinical trials justify more preclinical studies in order to clarify the underlying effects of EPO administration in septic induced-AKI focusing in a better understanding of the relevant processes that occur at the cellular level [14].

Previous studies in our laboratory have shown that EPO causes renoprotective effects through the amelioration of tubular renal apoptosis and VEGF/VEGFR-2 modulation in experimental sepsis [15,16]. However, whether EPO might ameliorate the renal microvascular injury or not; and the underlying molecular mechanisms of EPO renoprotection during septic AKI progression remains to be elucidated.

The aim of the present study was to describe the effects of rhEPO on the microvascular injury in the complex context of renal hypoxia and inflammation during LPS-induced AKI progression, through the assessment of EPO-R, HIF-1 alpha, iNOS, phospho NF- kB p65 and proangiogenic-related molecules (VEGF, VEGF-R2, and PeCAM-1).

2. Material and methods

2.1. Animals

Male Balb/c mice (22–25 g, age: 6–8 weeks) from the animal facility of the National Northeast University were housed in a controlled environment (22 ± 2 °C and relative humidity 55 ± 15%) with a 12-h light/dark cycle. Mice were allowed access to pelleted food and water ad libitum. All procedures involving them were conducted in compliance with the Guide for the Care and Use of Laboratory Animals (National Institute of Health, Bethesda, MD, USA) and the guidelines established by the Animal Ethical Committee of the Medical School of the National Northeast University (CICUAL-Med UNNE).

2.2. Experimental design

Animals were randomly divided into 4 groups of six mice each. They were treated as follows: (I) Control group: sterile saline solution (i.p.), (II) EPO group: 3000 UI/kg of recombinant human erythropoietin (Hemax, BioSidus, Argentina) in 2 subcutaneous doses 8 h apart; (III) LPS group: 8 mg/kg i.p. LPS (E. coli, 0127: B8; Sigma, St Louis, MO); (IV) LPS + EPO group, 8 mg/kg i.p. LPS dose followed by a 3000 UI/kg dose of EPO an hour later, administered as previously described in group (II).

The final dose of LPS, the timing and the route of EPO administration were adjusted according to preliminary work as previously described [15].

At each time for the experimental protocol (0, 12, 24 and 48 h) post LPS administration, mice were anesthetized (60 mg/kg pentobarbital i.p.) and bled by heart puncture. After being sacrificed by cervical dislocation, the kidneys were quickly excised and washed with cold saline solution. Renal samples were taken for routine histological, immunoblottings and immunohistochemical assays and serum samples were used in routine biochemical assays.

2.3. Assessment of renal function

Serum creatinine (Scr) and blood urea nitrogen (BUN) were determined by a Synchron CX7 autoanalyzer (Beckman, CA).

2.4. Histological examination and morphometric analysis

Formalin-fixed slides (4 μm) were stained with Hematoxylin and Eosin (H/E). The tubular injury was graded semiquantitatively as previously described [15]. Representative images were taken using an Olympus Coolpix-micro digital camera fitted on a CX-35 microscope (Olympus, Japan). Glomerular morphometric analyses for Bowman´s space area and the tubular cell height (μm) were performed using Image J software 1.50i (National Institutes of Health, Bethesda, MD). In ten cortical high-power fields (x400), at least 50 glomeruli were evaluated and calculated the mean area (μm2); while tubular cell heights (μm) were assessed randomly in ten high power fields.

2.5. Immunohistochemistry

Paraffin-embedded sections were, deparaffinised and rehydrated in graded alcohols using routine protocols, as previously described [17]. Briefly, sections were incubated with a rabbit polyclonal PeCAM-1 (Santa Cruz Biotechnology CA, USA, dilution 1:100), VEGF(Santa Cruz Biotechnology CA, USA, dilution 1:100), phosphorylated NF-κB p65 (Santa Cruz Biotechnology CA, USA, dilution 1:100), anti- iNOS (Cayman Chemicals, MI, USA, dilution 1:50) and VEGFR-2 (Cell Signaling Technology, Beverly, MA, USA, dilution 1:50) antibodies for 18 h at 4 °C. Positive controls used in IHC were: tonsil for PeCAM-1, normal murine kidney for VEGF, human cervical carcinoma for NF-kB p65, airway epithelial cells from lung tissue for iNOS, and human clear cell renal cell carcinoma for VEGF-R2.

Immunostaining was performed using a DAKO LSAB + /HRP kit (Dako Cytomation) followed by the application of a chromogen DAB (DAKO kit) according to the manufacturer´s instructions. Negative control samples were processed in PBS. Slides were then counterstained with hematoxylin and visualized under a light microscope. Images were taken using an Olympus Coolpix-micro digital camera fitted on a CX-35 microscope (Olympus, Japan). The percentage of positive areas for iNOS, VEGF, VEGFR-2, and phosphorylated NF-κB p65 was measured using the Image J software (National Institutes of Health, Bethesda, MD). Ten randomly selected cortical fields per cross-section were viewed (x400 original magnification).

2.6. Western blot analysis

EPO-R (M-20, sc-697 Santa Cruz Biotechnology CA, USA) and iNOS (Cayman Chemicals, MI, USA) expressions were determined by immunoblotting of cytosolic renal extracts, as previously described. Briefly, cortical renal sections were homogenized and lysed in an ice-cold buffer [10 mM HEPES pH 7.4, 10 mM KCl, 1.5 mM MgCl2, 0.5 mM dithiothreitol, 0.1% IGEPAL (Sigma Co, MO, USA)], supplemented with a protease inhibitor cocktail. Cell lysates were centrifuged at 14.000 *g* for 20 min and the supernatant (cytosolic fraction) was used for immunoblottings. Positive controls used in Western blottings were: cortical lysates from murine normal kidney for EPO-R and LPS-treated mouse spleen tissue lysates for iNOS.

The nuclear pellets were gently resuspended with ice-cold–wash buffer (20 mM HEPES pH 7.4, 1.5 mM MgCl2, 420 mM ClNa, 25% glycerol, 0.2 mM EDTA [ethylene glycol-bis (beta-amino ethyl ether)-N, N, N', N'–tetra acetic acid], 0.5 mM DDT), 0.2 mM PMSF with protease inhibitors and incubated for 1 h at 4 °C. Samples were centrifuged at 16.000 *g* at 4 °C for 30 min and supernatants were collected as nuclear extracts for HIF-1α immunoblotting. Positive control for HIF-1α expression were performed using nuclear cortical renal extracts from mice submitted to 6 h of hypoxia in a hypobaric chamber (simulated altitude of 0.6 atm).

Cytosolic and nuclear proteins (40 μg) were fractioned using 12% or 8% SDS-PAGE, respectively and then transferred to nitrocellulose membranes (Bio-Rad, CA, USA). Membranes were incubated with 1:500 dilutions of primary antibodies for iNOS (Cayman Chemicals, MI, USA) and HIF-1α (Novus Biologicals, Littleton, CO). As a positive controls β actin (Sigma-Aldrich) was used as internal loading control. Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson Immunoresearch Inc, USA). Immunocomplexes were

detected by an Opti4CN kit (Bio-Rad, CA, USA). Band optical density (OD) was determined using NIH-image software and results were expressed as the ratio: (protein of interest OD / β-actin OD) x100. Experiments were performed in triplicate and representative blots are shown.

2.7. Statistical analysis

Results were expressed as the mean ± standard deviation (SD). Comparisons between groups were performed using one-way analysis of variance (ANOVA) with a posthoc Bonferroni test correction. Data were analyzed with a Prism 4.0 software package (GraphPad Software Inc., San Diego, CA). Differences between groups were considered to be statistically significant at $P < 0.05$.

3. Results

3.1. EPO ameliorates renal dysfunction in LPS- induced AKI

LPS-induced AKI model was confirmed by the disturbance of biochemical parameters of renal dysfunction and histopathology evaluation, Fig. 1A shows a significant blood urea nitrogen (BUN) enhancement from 12 h post LPS that reached maximum levels at 48 h of the study (8 fold over control) suggesting an intense protein catabolism. Moreover, serum creatinine (Scr), also increased from 12 h (Fig. 1B) revealing the decrease of glomerular filtration rate. EPO treatment ameliorated the renal dysfunction since both parameters (BUN and Scr) decreased significantly at 12 h in the time course study ($P < 0.001$).

3.2. EPO attenuates histopathological damage in kidneys of endotoxemic animals

In order to evaluate the effects of EPO in renal tissue from mice following LPS-induced AKI, the histopathologic assessment was performed at each time of the experimental protocol. Fig. 2 illustrates the morphometric analyses of the urinary space of Bowman (Fig. 2A) among the studied groups during septic AKI progress. Main alterations included the strengthening of glomeruli with the consequent increment in the measurements of the urinary Bowman's space from 12 h compared to control samples ($P < 0.001$) suggesting glomerular ischemic injury (Fig. 2B). EPO treatment induced a reduction of these parameters from 24 h ($P < 0.001$) revealing an attenuation of glomerular ischemic damage induced by LPS administration.

On the other hand, the morphometric analyses of tubular renal injury in LPS induced AKI involved measurements of the tubular cell height. Fig. 3 illustrates differences among experimental groups during the progress of septic AKI. The height of tubular cells decreased from 12 h post LPS injection compared to control group ($P < 0.05$) suggesting the loss of brush border and tubular atrophy (Fig. 3A and B). EPO administration caused a significant attenuation of tubular atrophy from 24 h when these parameters were compared with the endotoxemic group ($P < 0.001$).

3.3. EPO enhances EPO-R expression in LPS-induced AKI

It is well known that EPO-R is widely expressed in glomerular, tubular and mesangial cells. In order to evaluate changes of EPO-R expression during LPS-induced AKI progress, Western blotting was performed at each time of the experimental protocol.

Immunoblots revealed that EPO-R expression decreased at 12 h post LPS ($P < 0.05$) and then returned to control levels (Fig. 4A). EPO administration caused a significant enhancement in the EPO-R expression from 12 h of the study ($P < 0.001$), that reached a maximum at 24 h and remained elevated until the end of the experience ($P < 0.001$).

3.4. EPO reduces HIF-1 alpha, iNOS and NF−κB expressions in LPS-induced AKI

LPS-induced AKI is associated with renal microvascular hypoperfusion and the subsequent tissue hypoxia. It is also known that there is a close interplay between hypoxic microenvironment, inflammation and endothelial dysfunction that contribute to the AKI progression in septic conditions.

In order to evaluate the effects of EPO administration on the aforementioned events, HIF-1 alpha, iNOS and phosphorylated NF−κB p65 expressions were assessed by immunoblotting and/or IHC.

The expression of hypoxia-inducible transcription factor 1-alpha (HIF-1α) was determined in nuclear extracts by Western blotting (Fig. 5). LPS administration significantly increased the expression of HIF-1α from 12 h to 24 h after LPS injection. EPO treatment caused the decrease of this master transcription factor expression, showing HIF-1α engagement in attenuating the hypoxic insult triggered by the endotoxemia.

On the other hand, the inducible nitric oxide synthase (iNOS) expression in kidney was determined by WB and IHC, as this enzyme is involved in inflammation and endothelial injury during endotoxemia induced-AKI. Immunoblottings revealed a marked increase of iNOS expression at 24 h post LPS administration (Fig. 6A). EPO administration significantly decreased the expression of iNOS from 12 h until the end of the experimental study ($P < 0.001$ vs. LPS Group).

iNOS expression pattern was determined by IHC at 24 h after LPS injection, revealing a significantly enhancement of immunostaining mainly located in the cytoplasm of proximal and distal tubular cells. EPO + LPS samples showed less iNOS immunoreactivity, associated with the almost total disappearance of perivascular infiltrate and interstitial leukocytes (Fig. 6B). Morphometric analyses of iNOS reinforce immunoblotting findings (Fig. 6C).

Otherwise, NF−κB mediates the transcription of a large number of genes, which products are known to play crucial roles in septic

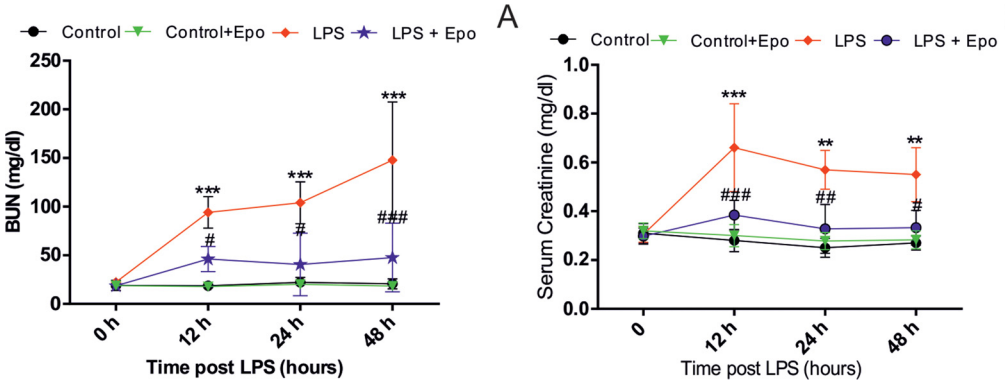

Fig. 1. Effects of EPO administration in renal functional parameters during LPS-induced AKI. (A) BUN (blood urea nitrogen) and (B) creatinine serum levels (mg/dl). Values are the mean ± SD (n = 6 mice / each group). ** $P < 0.01$ and *** $P < 0.001$ inidacates significant differences between LPS vs Control groups; # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ indicates significant differences between LPS and LPS + EPO groups.

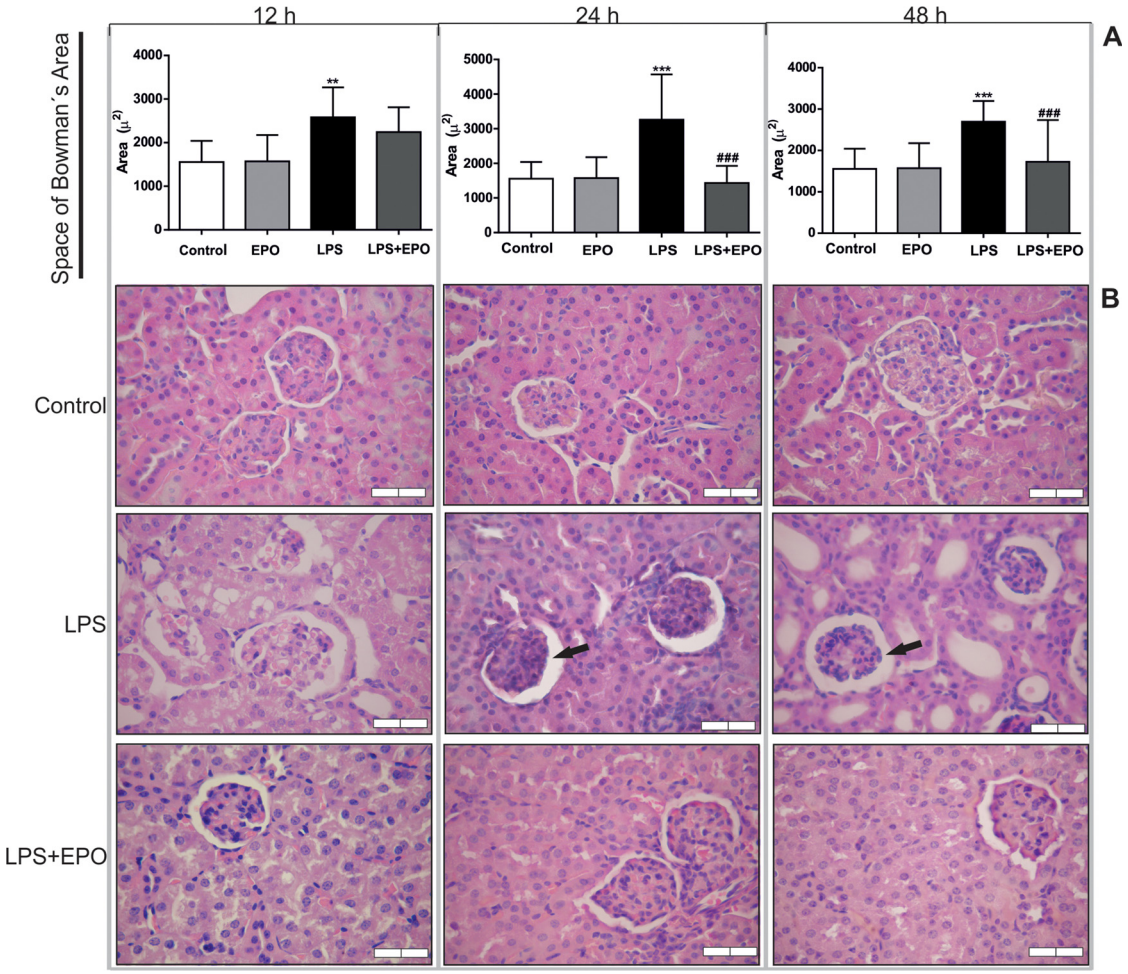

Fig. 2. Effects of EPO administration in glomerular histopathological damage in LPS-induced AKI. (A) Morphometric analyses of glomerular Bowman's space area (μm^2). (B) Representative images of glomeruli from renal cortical samples stained with H&E. Arrows indicates the enhancement of Bowman's space. Total magnifications x400. Lines represent 50 μm. Bar graphs represenent the mean ± SD among each experimental group at 12, 24 and 48 h of the expeirmental schedule. ** $P < 0.01$ and *** $P < 0.001$ indicates significant differences between LPS vs Control groups. ### $P < 0.001$ inidicates significant differences between LPS and LPS + EPO groups.

pathophysiology.

In order to determine whether EPO affects NF-κB activity, we studied the expression of phosphorylated NF-κB p65 by IHC at 24 h post LPS injection. Results showed focal intense immunoreactivity with nuclear and cytosolic patterns of staining in proximal convoluted tubular cells (Fig. 7A). Fig. 7B, illustrates that EPO administration attenuated the immunostaining for phospho NF−κB p65 (LPS vs LPS + EPO, $P < 0.01$). Thus, our findings suggest that EPO may reduce NF−κB signalling pathway activation via the inhibition of the nuclear translocation of this factor.

3.5. EPO promotes angiogenesis and ameliorates microvascular damage in the LPS-induced AKI

Microvasculature injury in LPS induced-AKI, is a direct consequence of hypoxic stress and the combined action of pro-inflammatory cytokines. Therefore, to assess the effect of EPO administration on angiogenesis, we studied the expression of the vascular endothelial growth factor (VEGF), the vascular endothelial growth factor receptor isoform 2: VEGF-R2 (KDR or Flk-1) and a microvasculature marker protein: PeCAM-1 (CD31).

Since that the maximum expression of HIF-1α was detected at 12 h after LPS injection, the expressions of VEGF, VEGFR-2, and CD31 were determined by IHC at this experimental time.

The expression of VEGF was extensively expressed in control group, and it was mainly located in proximal and distal tubular cells (Fig. 8A, Panel I). However, immunostaining of this factor was considerably reduced in LPS-treated animals, particularly in areas with cellular damage or tubular atrophy (Panel II). Furthermore, treatment with EPO significantly enhanced the expression of this proangiogenic factor (Panel III) ($P < 0.01$ vs LPS group). These findings were verified by morphometric analyses (Fig. 8B).

In order to assess whether EPO administration affects the expression of the main VEGF receptor, VEGFR-2, the localization of this protein was evaluated by IHC, 12 h post LPS challenge (Fig. 9).

The expression of VEGFR-2 was mostly observed in peritubular capillaries or evenly in the glomerular capillary tuft in control mice, although LPS challenge caused a marked decrease in its expression. EPO administration produced the recovery of VEGF-R2 levels closely to controls in endotoxemic mice (Fig. 9A). Morphometric analyses of VEGF-R2 positive areas reinforce these outcomes (Fig. 9B).

On the other hand, the platelet endothelial cell adhesion molecule-1 (PeCAM-1/CD31), involved in the endothelial cell intercellular junctions, has been reported to have proangiogenic activities [18]. Hence, the putative protective effect of EPO administration on septic renal microvasculature was assessed by IHC at 12 h after LPS injection.

PeCAM-1 immunostaining revealed that it was mainly expressed in peritubular endothelium, glomeruli and in the basal membrane of

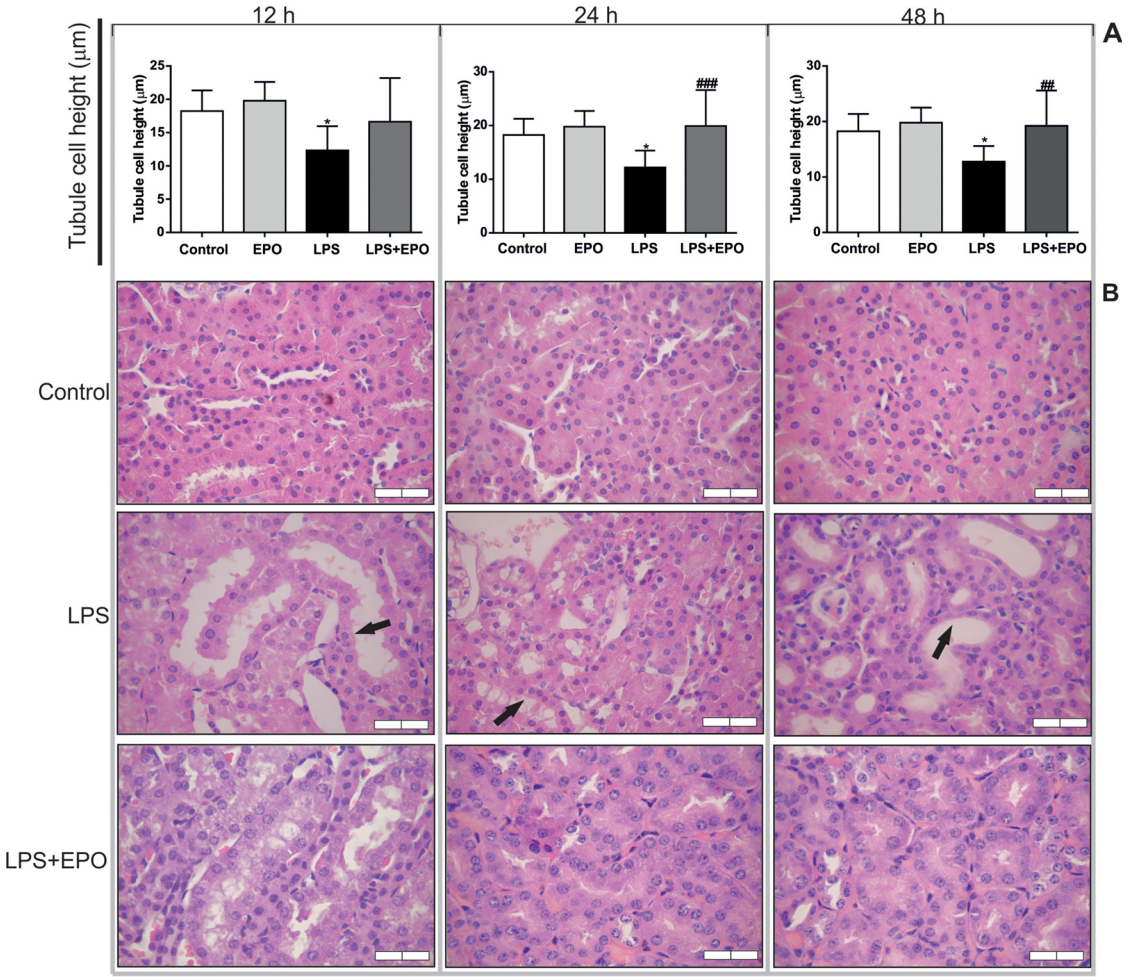

Fig. 3. Effects of EPO administration in tubular histopathological damage in LPS-induced AKI.
(A) Morphometric analyses of tubular heights (μm). (B) Representative images of tubules from cortical sections stained with H&E. Arrows indicate the reduction of tubular cells height. Total magnification x 400. Lines represent 50 μm. Bar graphs represent the mean ± SD among each experimental group at 12, 24 and 48 h of the experimental schedule.* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ indicate significant differences between LPS vs Control groups. ## $P < 0.01$ and ### $P < 0.001$ indicate significant differences between LPS and LPS + EPO groups.

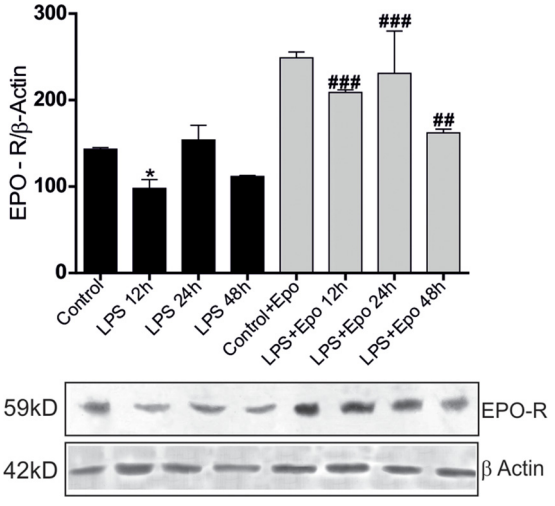

Fig. 4. Effects of EPO administration on EPO-R expression in LPS-induced AKI. Western blottings of EPO-R in LPS induced-AKI. β- actin was used as internal control. A representative blot of three independent experiments is shown. Values are the mean ± SD (n = 6 mice/each group). *$P < 0.05$ indicates significant differences between LPS vs Control groups. ## $P < 0.01$ and ### $P < 0.01$ indicate significant differences between LPS and LPS + EPO groups.

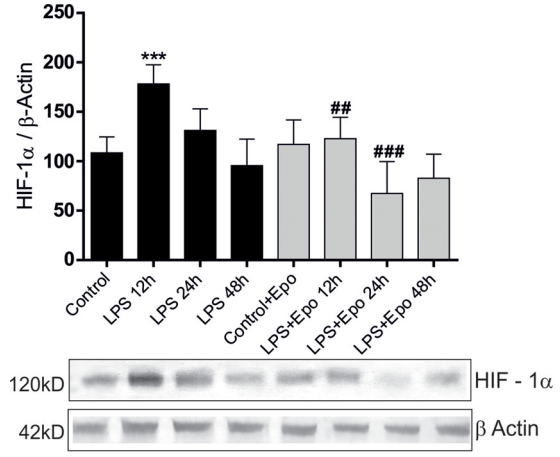

Fig. 5. Effects of EPO administration on HIF-1α expression in LPS-induced AKI. HIF-1α immunoblotting. β-actin was used as internal control. A representative blot of three independent experiments is shown (n = 6 mice/each group). Results are expressed as arbitrary densitometry units (mean ± SD). ** $P < 0.01$ and *** $P < 0.001$ LPS vs. control groups; ## $P < 0.01$ and P ### < 0.001, LPS vs. LPS + EPO groups.

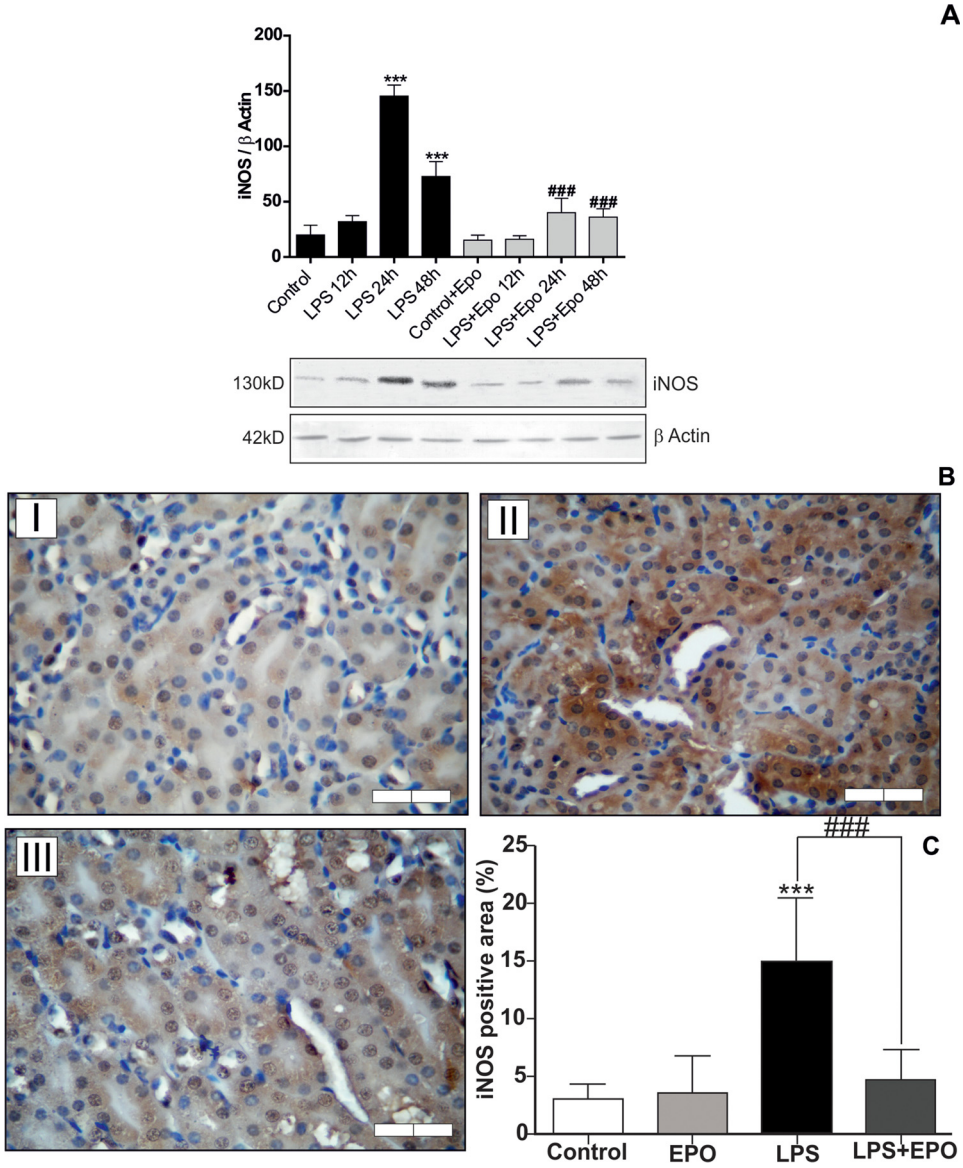

Fig. 6. Effect of EPO administration on iNOS expression in LPS-induced AKI.
(A) Immunoblotting of renal iNOS in total cortical lysates. β-actin was used as internal control. These results are representative of three independent experiments and are expressed as arbitrary densitometry units. (B) Immunohistochemistry of iNOS, 24 h after LPS injection. Representative micrographs corresponding to: I) Control group. II) LPS group. III) LPS + EPO group. Total magnification 400×. The bars represent 50 μm. (C) Morphometric analyses of iNOS positive areas. Results are expressed as mean ± SD. *** $P < 0.001$ Control vs. LPS groups. ### $P < 0.001$ LPS groups vs. LPS + EPO groups.

Bowman's capsule (Fig. 10). However, PeCAM-1 expression decreased strongly in endotoxemic renal sections and exhibited a minimal diffuse immunoreactivity in renal endothelial cells and glomeruli. EPO treatment restored PeCAM-1 expression indicating its role as renal protective agent through its pro-angiogenic effect.

4. Discussion

The pathogenesis of sepsis-associated AKI is currently seen as a multifactorial process involving apoptotic, immune, and inflammatory events that aim to focus on novel potential therapeutic agents [19]. However, the interplay between inflammation, the oxidative stress, microvascular dysfunction, and the adaptive response of the tubular epithelial cell to the hypoxic insult in septic AKI is a source of much debate [20].

To the best of our knowledge, this the first study that demonstrates the attenuation of renal microvascular damage by rhEPO during septic-AKI progression, through the assessment of EPO-R, HIF-1 alpha, iNOS, phospho NF-kB p65 and proangiogenic-related molecules (VEGF, VEGF-R2, and PeCAM-1).

In the present study, murine AKI model has been successfully established by treating mice with LPS. Animals presented a substantial kidney injury with obvious changes of histopathology and serum biochemical index of renal injury according to previous studies [21,22].These renal functional alterations, as well as, the histopathological abnormalities were partially reversed by EPO treatment, reinforcing previous reports [15,23,24].

It is widely known that EPO exerts its biological functions through EPO-R. In this study, blotting results showed that EPO caused a statistically significant over expression of EPO-R since 12 h of the experimental protocol until the end of the experience, in accordance with a previous report [25].

On the other hand, it has been reported that sepsis affects several aspects required for a proper microvascular function, thereby resulting in significant disturbances in capillary perfusion [26]. Indeed, the present experimental data revealed that HIF-1 α expression, the main hypoxia master regulator protein in low oxygen tension adaptation, increased between 12–24 h post LPS challenge. The enhancement of HIF-1 α was coincident with tubular and glomerular alterations, most of which, were related to ischemia. These results show that renal hypoxia occurred during endotoxemia and there is a strong involvement of HIF-1α in the pathogenesis of sepsis-induced AKI in accordance with Schaalan & Mohamed (2016) in septic patients [27].

Septic microvascular injury involves the deregulation of pro-

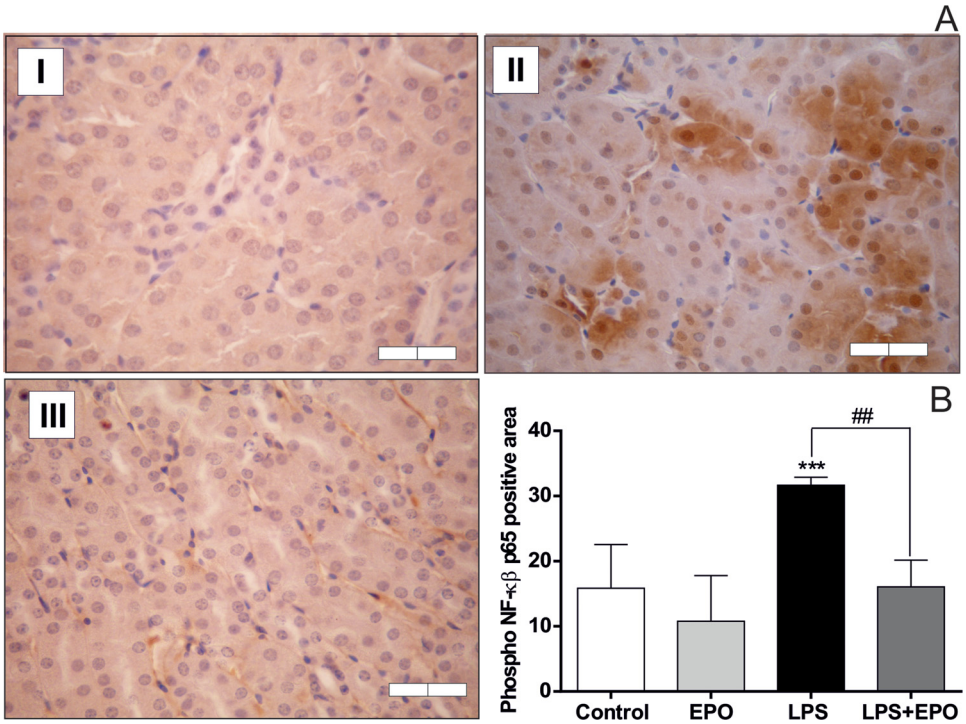

Fig. 7. Effect of EPO administration on NF − κB expression in LPS-induced AKI.
(A) Immunohistochemistry of phosphorylated NF − κB p65, 24 h post LPS injection. Representative micrographs corresponding to: I) Control group. II) LPS group III) LPS + EPO group. Total magnification 400 x. The bars represent 50 μm. (B) Morphometric analyses of phospho NF − κB p65 positive areas. Results are expressed as mean ± SD. *** $P < 0.001$ Control vs. LPS groups. ## $P < 0.01$ LPS vs. LPS + EPO groups.

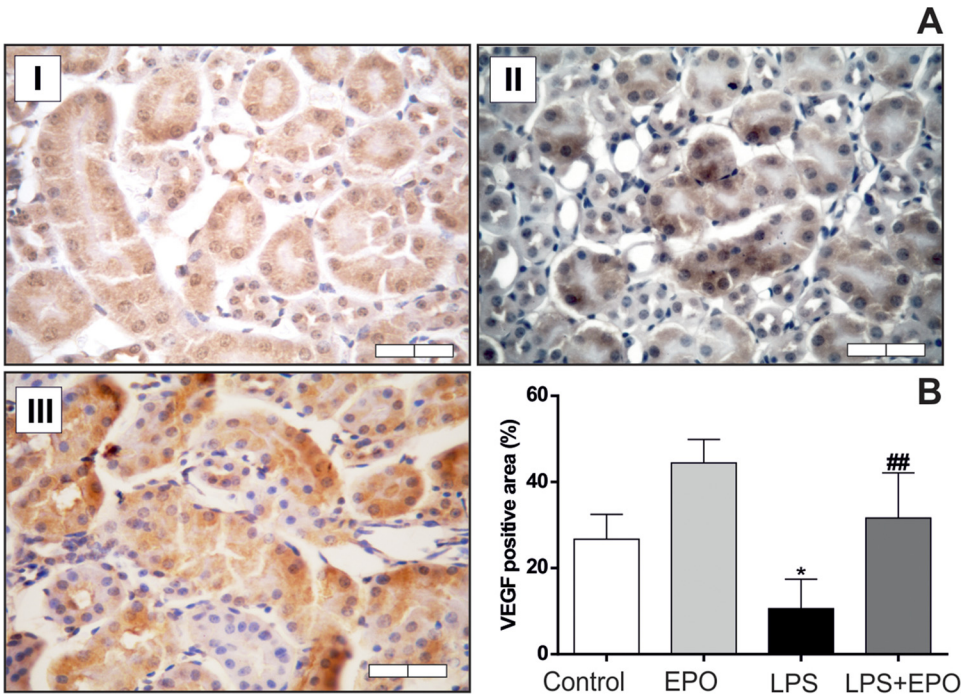

Fig. 8. Effect of EPO administration on VEGF expression in LPS-induced AKI.
(A) Immunohistochemistry of VEGF 12 h post LPS injection. Representative photographs of: I) Control group; II) LPS group; III) LPS + EPO group. Total magnification x 400. Lines represent 50 μm. (B) Morphometric analysis of VEGF positive area. Results are expressed as mean ± SD. * $P < 0.05$ Control vs. LPS groups. ## $P < 0.01$ LPS vs. LPS + EPO groups.

angiogenic factors such as VEGF. The pair VEGF /VEGF-R2 stimulates the proliferation, migration, and survival of endothelial cells and promotes the vascular permeability [28]. Furthermore, VEGF has been described as a survival factor for proximal tubular cells and critical for maintenance of renal vasculature during kidney damage including AKI [29]. Our results demonstrated that endotoxemia decreased renal VEGF expression in cortical tubules at 12 h post LPS in agreement with previous reports [16,30]. Shown to be a downstream target of HIF-1α, VEGF expression should be increased after HIF-1α induction. However, we observed a reduction in VEGF expression in LPS induced-AKI, despite an increased expression of HIF-1α. This apparent controversy is based on the fact that VEGF transcription is also regulated by a variety of hormones, growth factors and cytokines (i.e. TGF β, insulin-like growth factor 1, angiotensin II, IL-1 and IL-6) [31].

Regarding VEGFR-2 occurrence in septic AKI, Xu et al. [32] communicated that the renal VEGFR-2 expression did not show significant variations following endotoxemia. In contrast, our findings showed that VEGFR-2 expression was notably decreased in peritubular capillaries at 12 h post LPS. Our experimental data are in accordance with in vitro studies that reported the decrease of VEGFR-2 expression due to pro-inflammatory cytokines [33]. These results were also confirmed using an in vivo model of septic acute lung injury [34]. Additionally, the decrease of VEGF/VEGFR-2 expressions post LPS was coincident with the notable reduction of PeCAM-1 expression, which was noticed in

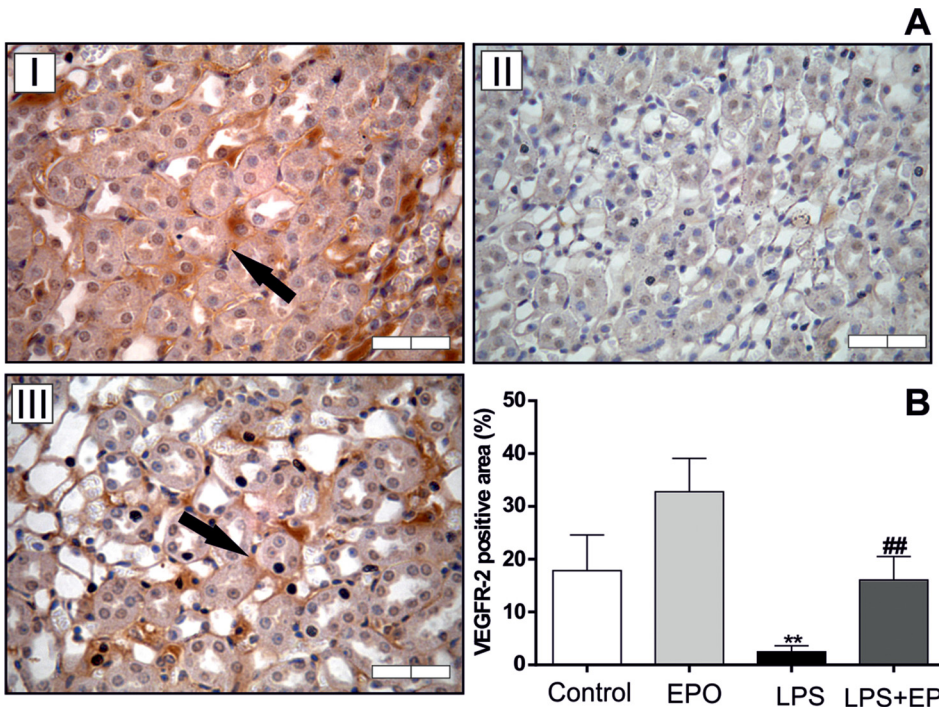

Fig. 9. Effect of EPO administration on VEGFR-2 expression in LPS-induced AKI.
(A) Immunohistochemistry of VEGFR-2. Representative photographs of I) Control group; II) LPS GroupIII) LPS + EPO group. Total magnification 400 × . Lines represent 50 μm. (B) Morphometric analysis of VEGFR-2 positive area. Results are expressed as mean ± SD. ** P < 0.01 Control vs. LPS group. ## P < 0.01 LPS group vs. LPS + EPO group.

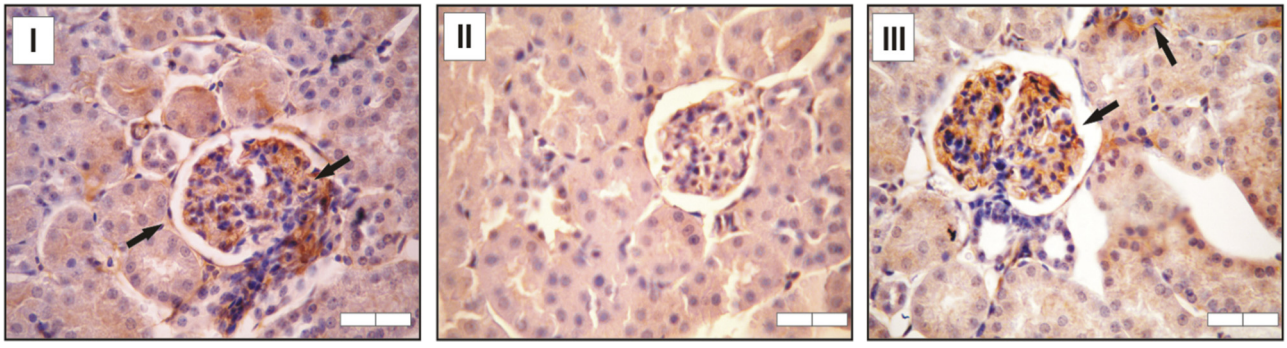

Fig. 10. Effect EPO administration on PeCAM-1 expression in LPS induced AKI. Immunohistochemistry of PeCAM-1, 12 h after LPS injection. Representative photographs of the PeCAM-1 immunostaining: I) Control group; II) LPS group; III) LPS + EPO group. Total magnification 400 × . Lines represent 50 m.

peritubular and glomerular endothelium as it has been reported by Cichy et al. [35].

EPO can exert renoprotection by amelioration of hypoxia and enhances microvascular cell survival and/or angiogenesis in another experimental model of renal injury [36]. However, these effects in endotoxemic AKI are not completely dilucidated.

Our data revealed that, in this experimental model, EPO treatment mediated the attenuation of renal hypoxia as supported by the decrease of HIF-1α expression. Additionally, EPO ameliorated the renal microvascular injury through VEGF over expression and the restoration of VEGF-R2 and PeCAM-1 immunoreactivities.

Hence, our findings suggest that EPO/EPO-R system plays an important role in angiogenesis through the up regulation of VEGF/VEGFR-2 pair, indicating that both systems are engaged in renal microvascular protection during septic AKI. The interplay between both signaling pathways in angiogenesis was also reported using a murine model of systemic ischemia [37].

Sepsis induced-microcirculatory dysfunction has been linked to several factors, including vasoconstrictor and vasodilator mediator imbalances. The nitric oxide (NO) system is a major contributor to this endothelial injury. Moreover, the induction of nitric oxide synthase (iNOS) and the generation of oxygen radicals during sepsis cause peroxynitrite-related tubular injury [38].

In this study, renal iNOS expression significantly increased from 24 h after LPS administration, revealing a strong immunoreactivity in the cytoplasm of proximal renal tubules in accordance with a previous report [39]. Furthermore, it has been shown that HIF-1α triggers the induction of iNOS in several cell types[40]. Our results are in line with these outcomes since the maximal iNOS expression at 24 h post LPS occurred after HIF-1α overexpression. These results reinforce the concept that iNOS plays a key pathophysiological role in endotoxemic induced renal hypoxia and may participate in the organ dysfunction by extending the microvascular injury [39]. Additionally, EPO has also been shown to inhibit the activity of iNOS in a variety of experimental settings [41,42]. In this study, the EPO treatment caused a significant decrease of iNOS expression in the renal cortex. Our data are in line with previous studies showing that EPO inhibited iNOS expression, thereby attenuating cytotoxicity and vascular dysfunction during inflammation [7,24,42].

NF-κB is one of the most important and widely used transcription factors and is involved in the regulation of gene expression in LPS-induced inflammation response during kidney injury and pathophysiology of sepsis. The activation of NF-κB is regulated by IκB and IKKs, which can be activated by several stimuli such as lipopolysaccharide. Experimental data suggest that LPS triggered the phosphorylation of NF-κB p65 in accordance with Fan et al [43].

Furthermore, it has been reported that HIF-1α is related to the inflammatory events through the enhancement of NF-kB signaling with HIF stabilization mediated by Toll-like receptors (TLR-2 and TLR-4) and the potentiation of NF-kB signaling through the regulation by the HIF-PHDs [44]. Besides, there is evidence for the linkage of LPS mediated NF-κB signaling and the HIF pathway in renal tissue. Consequently, our experimental data showed the simultaneous overexpression of HIF-1α and phosphor NF- κB p65 at 24 h post LPS insult.

The rhEPO treatment restrained the phosphorylation of the NF-κB p65 suggesting that it exerts renoprotective effect, in part, by the attenuation of the inflammatory response, in accordance with Coldewey et al [45].

In conclusion, the current study support that EPO treatment can ameliorate kidney injury in LPS-induced AKI by decreasing renal microvascular injury, reducing renal inflammatory response and improving tissular oxygenation. These protective effects depend on EPO-R over expression, the subsequent increase of proangiogenic-related molecules (VEGF, VEGF-2, and PECAM-1) and the decrease of HIF-1 alpha, i-NOS, and NF-κB expressions. Further studies will be required to confirm the underlying relationships among the molecular mechanisms associated with these effects.

Finally, this study provides new insights into the renoprotective mechanisms of EPO in endotoxemic-AKI, suggesting that EPO may be a potential therapeutic agent to ameliorate microvascular dysfunction and to improve the prognosis of septic patients.

Conflict of interest

The authors declare no conflict of interest.

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