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Erythropoietin attenuates renal and pulmonary injury in polymicrobial induced-sepsis through EPO-R, VEGF and VEGF-R2 modulation

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ABSTRACT

Sepsis remains the most important cause of acute kidney injury (AKI) and acute lung injury (ALI) in critically ill patients. The cecal ligation and puncture (CLP) model in experimental mice reproduces most of the clinical features of sepsis. Erythropoietin (EPO) is a well-known cytoprotective multifunctional hormone, which exerts anti-inflammatory, anti-oxidant, anti-apoptotic and pro-angiogenic effects in several tissues. The aim of this study was to evaluate the underlying mechanisms of EPO protection through the expression of the EPO/EPO receptor (EPO-R) and VEGF/VEF-R2 systems in kidneys and lungs of mice undergoing CLP-induced sepsis.

Male inbred Balb/c mice were divided in three experimental groups: Sham, CLP, and CLP + EPO (3000 IU/kg sc). Assessment of renal functional parameters, survival, histological examination, immunohistochemistry and/or Western blottings of EPO-R, VEGF and VEGF-R2 were performed at 18 h post-surgery.

Mice demonstrated AKI by elevation of serum creatinine and renal histologic damage. EPO treatment attenuates renal dysfunction and ameliorates kidney histopathologic changes. Additionally, EPO administration attenuates deleterious septic damage in renal cortex through the overexpression of EPO-R in tubular interstitial cells and the overexpression of the pair VEGF/VEGF-R2.

Similarly CLP- induced ALI, as evidenced by parenchymal lung histopathologic alterations, was ameliorated through pulmonary EPO-R, VEGF and VEGF-R2 over expression suggesting and improvement in endothelial survival and functionality.

This study demonstrates that EPO exerts protective effects in kidneys and lungs in mice with CLP-induced sepsis through the expression of EPO-R and the regulation of the VEGF/VEGF-R2 pair.

1. Introduction

Sepsis is a severe condition associated with great mortality in intensive care patients and trauma victims. It is characterized by an infectious process that induces a serious systemic inflammatory response, which ultimately leads to organ failure. Mediators such as pro-inflammatory cytokines, reactive oxygen species, nitric oxide, are engaged in controlling the immune response and in consequence, regulate circulatory flow variations and tissue injury [1,2]. Sepsis is defined as severe if there is evidence of organ dysfunction [3] and those that fail most frequently in septic patients are the kidneys and the lungs [4].

Acute kidney injury (AKI) is a common outcome of sepsis and is responsible for a significant morbidity and mortality in renal patients [5]. Septic kidney shows acute tubular injury (ATI), characterized by renal tubular cell death and shedding of necrotic cells into the tubular lumen. Also, ischemia and inflammatory cytokines in addition with damage-associated molecular pattern molecules provokes a widespread tubular cell apoptosis. These changes, consequently, explain the eution to renal failure and finally, the loss of renal function [1].

Acute lung injury (ALI) and the more severe form, acute respiratory distress syndrome (ARDS) can be caused by several pathological processes that affect the lung. Among them, sepsis is a common cause that provokes indirect lung injury and is associated with the development of the ARDS [6].

The pathological process underlying this sequence is caused primarily by neutrophil-dependent and platelet-dependent damage to the endothelial and epithelial barriers of the lung. This

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allows fibrin-rich edema fluid, red blood cells, and neutrophils to enter the alveoli, and this influx leads to the inactivation of surfactant. These alterations collapse tissue and compromise lung function [7].

Several preclinical models have been developed to evaluate the effect of septic AKI and ALI. The cecal ligation and puncture (CLP) model is widely used because the resulting polymicrobial sepsis mimics many features of human sepsis [8,9].

Unfortunately, current specific options for treatment of ALI/ARDS and AKI are limited, although the complex pathology of sepsis offers an array of potential pharmacologic targets. The research of new therapeutic approaches should include the use of combined-agent treatments or a drug with multiple beneficial effects. In this regard, (EPO) has emerged as a multifunctional tissue-protective cytokine with anti-inflammatory, anti-apoptotic, pro-angiogenic and immunomodulatory properties [10].

One of the biological and biochemical activities of EPO that could be potentially applicable for AKI and ALI septic management might inve the modulation of the vascular endothelial growth factor (VEGF) and its main receptor (VEGF-R2).

VEGF is an endothelial and multifunctional cytokine that plays a central role in microvascular permeability and angiogenesis. The biological effects of VEGF are mediated by two receptors, namely VEGF-R2 (KDR or Flk-1) and VEGF-R1 (Flt-1) [11].

It is well-known that elevated serum levels of VEGF in sepsis is associated with disease severity and mortality [12]. Nevertheless, the role of the pair VEGF/VEGFR-2 related to EPO – mediated effects in septic AKI and ALI, is not completely understood.

Therefore, the aim of this study was to evaluate the effect of EPO exogenous administration in renal and lung murine injury following CLP –induced sepsis through the assessment of EPO/EPO-R and VEGF/VEGF-R2 systems.

2. Materials and methods

2.1. Animals

Male Balb/c mice (22–25 g, age: 6–8 weeks) were obtained from the animal facility of the National Northeastern University, Argentina. Animals were housed in a controlled environment ($22 \pm 2\,^{\circ}C$ and relative humidity $55 \pm 15\%$) with a 12-h light/12-h dark cycle. Mice were allowed access to pelleted food and water ad libitum. A total of 48 animals were used for all procedures (10 mice/group for survival assays and 6 mice/group for the other set of experiments). All procedures inving these animals were conducted in compliance to 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 Northeastern University. All surgeries were performed with a clean technique.

2.2. Surgery

Sepsis was induced by cecal ligation and perforation [13]. Briefly, a midline laparotomy was performed on a temperature-regulated table under ketamine/xylazine (50: 8 mg/kg i.p.) anesthesia. After externalization, the cecum was ligated below the ileocecal valve with 4-0 silk. Then, two perforations were made with a 20-gauge needle (antimesenteric edge). The abdomen was closed in two layers, followed by a 30 ml/kg s.c injection of sterile normal saline for rehidratation [14]. In sham-operated animals, the abdomen was opened but the cecum was neither ligated nor punctured. Mice were housed in individual cages and they were monitored for signs of discomfort throughout the recovery period.

2.3. Experimental design

For the experiments, animals were randomly divided into three groups of 18 mice each. They were treated as follows: (I) Sham group: sterile saline solution (i.p.), (II) EPO + CLP group: 3000 UI/kg of recombinant human erythropoietin (Hemax, BioSidus, Argentina) in 2 subcutaneous (s.c.) doses 7 h apart; (III) CLP group as previously described in 2.2 Section. Additionally, the timing and the route of EPO administration were performed as described by Aoshiba et al. [15].

At 18 h after surgery, mice (n = 6 each group) were anesthetized (60 mg/kg pentobarbital i.p.) and bled by heart puncture. After being sacrificed by cervical dislocation, the kidneys and lungs were quickly excised and washed in cold saline solution. Samples were taken for routine histological, immunoblottings and immunohistochemical assays. Additionally, serum samples were used in routine biochemical assays.

2.4. Survival study

Mice (10/group) were randomized into one of three experimental groups: Sham, CLP and CLP+ EPO. Survival was assessed at 8, 12 and 18 h post-surgical procedure in each group. Mice surviving 18 h appeared in stable conditions.

2.5. Assessment of renal function

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

2.6. Histopathological studies

For routine histological analysis, lungs or kidneys were fixed in phosphate-buffered formaldehyde, embedded in paraffin and stained with Masson, Hematoxylin/Eosin (H/E) and periodic acid-Schiff (PAS) techniques. Sections were examined with 400× magnification for renal and lung damage and ten cortical high-power fields were examined at random by two blinded observers. Histological criteria for renal damage were tubular cell degeneration/vacuolization, intratubular bleeding, loss of brush border, caryolysis and tubular dilatation/flattening. Alterations in affected tubules were graded as follows: 0, less than 5%; 1, 5–33%; 2, 34–66% and 3, over 66% [16]. The frequency of intratubular casts was quantified by counting at least 10 fields at ×400 PAS-stained sections. Lung sections were examined for the presence of hyaline membranes, diffuse and intra-alveolar haemorrhage and diffuse interstitial pneumonia. Ten high- power fields of lung parenchyma were graded on a scale of 0–3 for obtaining lung injury scores (0, absent and appears normal; 1, mild, 2, moderate; 3, intense). A mean score for each of the parameters was then calculated.

Images were taken with an Olympus Coolpix-micro digital camera fitted on a CX-35 microscope (Olympus, Japan).

2.7. Immunohistochemistry

Paraffin-embedded sections were, deparaffinized and rehydrated in graded alcohols using routine protocols, as previously described [17]. Briefly, sections were microwaved in citrate buffer (pH 6.0) for antigen retrieval and endogenous peroxidase activity was blocked in 3% H_2O_2 for 15 min. Subsequently, sections were incubated with a rabbit polyclonal anti-VEGF-R2 (Cell Signaling Technology, Beverly, MA, USA; dilution 1:100) for 18 h at $4\,^{\circ}C$. Immunostaining was performed using a DAKO LSAB+/HRP kit (Dako Cytomation) followed by the application of a chromogene DAB (DAKO kit) according to the manufacturer's instructions. Negative control samples were processed in PBS. Slides were then

counter-stained with hematoxylin and visualized under a light microscope.

2.8. Morphometric analysis

The percentage of positive areas for VEGF-R2 was measured using the Image J software (National Institutes of Health, Bethesda, MD). Ten randomly selected cortical fields per cross-section were viewed (×400 original magnification). Images were taken using an Olympus Coolpix-microdigital camera fitted on a CX-35 microscope (Olympus, Japan).

2.9. Statistical analysis

Statistics Survival curves were constructed by the Kaplan-Meer method and analyzed for differences using the score test of the Cox proportional hazards model for grouped data.

Results from Western blottings, immunohistochemistry and scorings from lung and renal injuries were expressed as mean ± standard error of mean (SEM). Comparisons between groups were performed using one- way analysis of variance (ANOVA) with a post hoc Bonferroni test correction. Data were analysed 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 improves survival and renal function in CLP model

To determine whether EPO reduces mortality owing to sepsis on a CLP model a survival test was performed. Fig. 1 illustrates the survival rates in the different experimental groups after 18 h of the surgical procedure without antibiotics administration. As expected, mice from CLP group suffered more than 85.0% of mortality compared to the Sham group. The administration of EPO reduced the mortality rates, allowing 80.0% and 72.5% of survival at 12 and 18 h, respectively. No deaths occurred in sham-operated animals.

Creatinine and blood urea nitrogen (BUN) levels were significantly enhanced in CLP treated animals compared to the sham group ($p < 0.001$) confirming the decrease of renal function induced by sepsis (Fig. 2). Conversely, CLP + EPO group displayed a significant improvement in those biochemical parameters. BUN values in CLP+ EPO mice were 0.25 times below CLP group

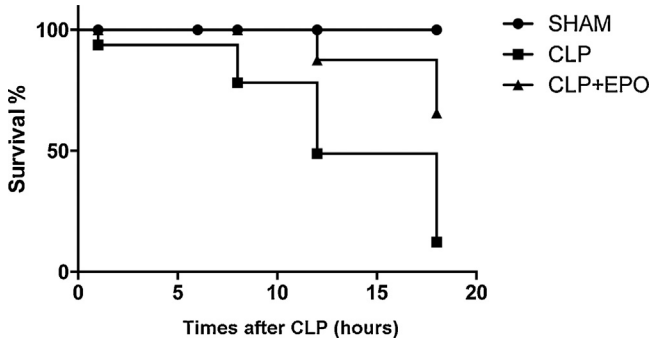

Fig. 1. Effect of EPO administration in mice survival in CLP-induced sepsis. Percentages of survival are represented for Sham, CLP and CLP + EPO experimental groups (n = 10 mice/each group). Kaplan-Meier survival analysis and log rank test were used.

($P < 0.01$); whilst serum creatinine decreased with the hormone administration 0.40 fold between the mentioned groups ($P < 0.001$).

3.2. EPO prevents histopathological damage in kidneys and lungs of animals undergoing CLP

In order to evaluate the effects of EPO in renal and lung tissues from mice following CLP-induced sepsis, several histopathologic staining techniques were performed (Masson, H/E and PAS). Fig. 3 exhibits the differences among renal histopathology in the studied groups. Briefly, the H/E stained renal slides from the CLP group Fig. 3A-II showed a moderate tubular renal injury as manifested by tubular cell desquamation and cell peeling, intratubular bleeding, caryolysis, cell vacuolization and loss of brush border compared to Sham Group (Fig. 3A-I). Notably, all these alterations were diminished in the EPO treated group (Fig. 3A-III) as indicated by the cortical injury scores obtained (Fig. 3B).

Masson's trichomic staining clearly reveals that EPO administration ameliorates histology of the fibrotic outcomes (blue colour) in the CLP renal samples. Periodic acid–Schiff (PAS) staining of kidney sections shows that the brush-border defects of tubular epithelial cells in CLP samples were restored with EPO exogenous administration. Moreover, EPO treatment reduced significantly the frequency of intratubular casts (Fig. 3B).

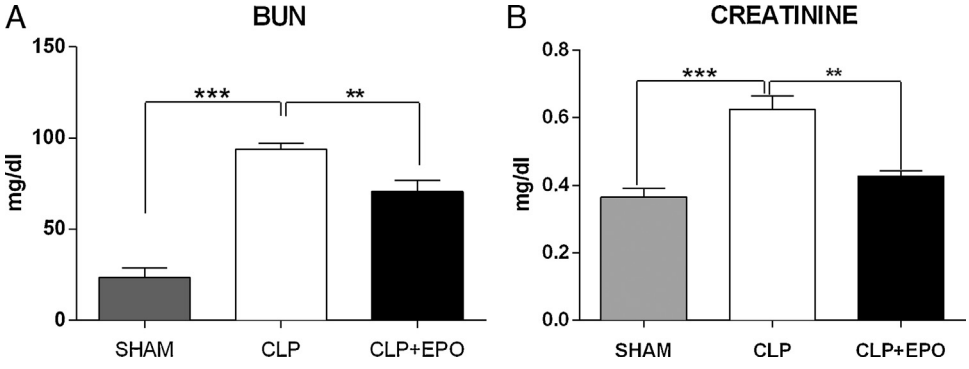

Fig. 2. BUN and creatinine levels as a measure of renal dysfunction in CLP-induced sepsis.
(A) BUN (blood urea nitrogen) and (B) creatinine serum levels (mg/dl) were measured in Sham, CLP and CLP + EPO samples (n = 6 mice/each group). Values are mean ± SEM. ** $P < 0.01$ and *** $P < 0.001$ indicate significant differences.

Similarly, the lung parenchyma of animals undergoing CLP showed morphological changes (Fig. 4A-II) such as the presence of hyaline membranes, diffuse and intra-alveolar haemorrhage and diffuse interstitial pneumonia. Additionally, a thickening of the interstitium and alveolar walls was observed.

Likewise, as it was observed in renal CLP-induced injury, the EPO exogenous administration in septic mice ameliorated lung histopathologic alterations (Fig. 4A-III), as indicated by the lung injury scores obtained (Fig. 4B).

3.3. EPO administration improves the EPO-R expression in renal and pulmonary tissues of septic animals

The expression of EPO-R following EPO administration was assessed in kidneys and lungs of each experimental group by immunoblotting. Western blots revealed that EPO-R expression in the CLP + EPO treated group, exhibited a significant enhancement when compared to the CLP treated group ($P < 0.01$). There was no statistically significant difference in EPO-R expression between CLP and Sham groups. The EPO-R expression in lung homogenates of the CLP group was significantly less than the high EPO-R levels detected in the Sham group. EPO administration enhanced the EPO-R expression (Fig. 5).

3.4. Effects of EPO on VEGF and VEGFR-2 expressions in renal and pulmonary tissues of septic animals

In order to evaluate the effect of EPO administration on angiogenesis, and also its possible mechanism of action in this experimental model, expressions of vascular endothelial growth factor (VEGF) and its main endothelial receptor (VEGF-R2; KDR o Flk-1) were analysed in both tissues (kidneys and lungs).

Statistical significant differences were observed between the CLP group and the CLP + EPO group in the expression of VEGF by western blotting in renal and pulmonary homogenates (Fig. 6A, $P < 0.001$).

Fig. 6B-I shows that the expression of VEGFR-2, in kidneys of control animals was mainly observed at vascular endothelial cells in the renal glomerulus and in the apical region of the proximal and distal conuted tubular cells. A marked decrease in the expression of this receptor was noticed in CLP samples. Notably, the CLP + EPO samples showed the recovery of the VEGFR-2 receptor expression,

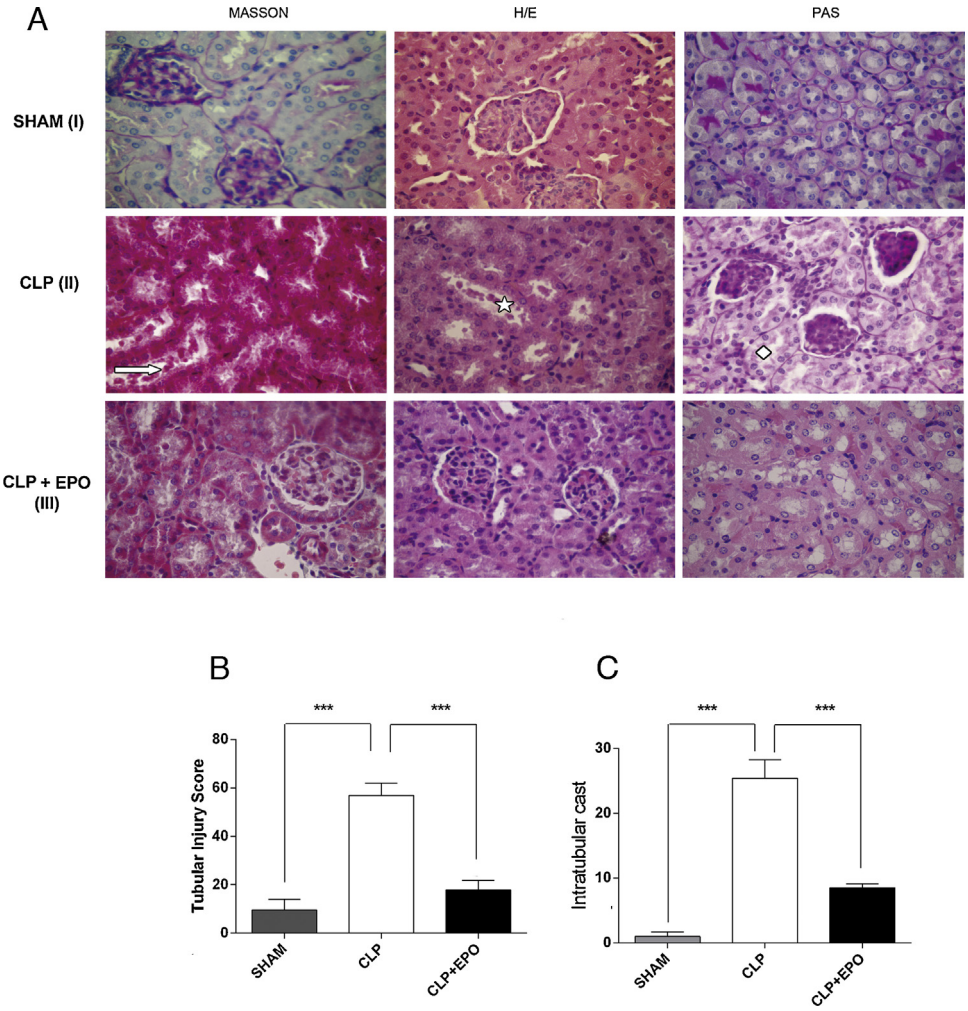

Fig. 3. Effects of EPO administration on renal morphology in CLP-induced sepsis.
(A) Representative Masson, PAS and H/E stained renal sections from Sham (I), CLP (II) and CLP + EPO (III) animals are shown. Original magnification × 400. CLP caused moderate tubular injury as manifested by tubular desquamation and brush border loss (arrow); intratubular bleeding (star) and tubular cell vacuolization (diamond). EPO treatment ameliorated these histopathological changes.
(B) Scoring of tubular injury. Scores were measured in Sham, CLP and CLP + EPO samples (n = 6 mice/each group). Values are mean ± SEM. *** $P < 0.001$ indicate significant differences.
(C) Frequency of intratubular casts. Casts were counted in Sham, CLP and CLP+ EPO sections stained with PAS (n = 6 mice/each group). Values are mean ± SEM. *** $P < 0.001$ indicates significant differences.

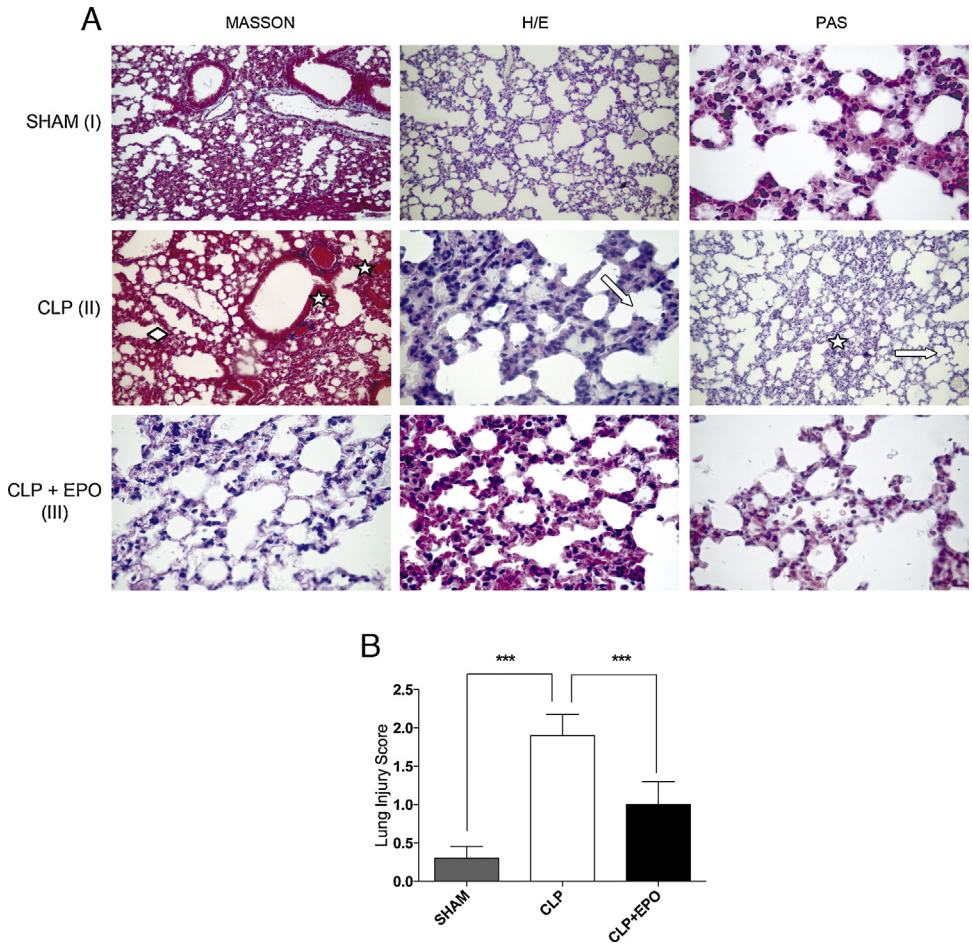

Fig. 4. Effects of EPO administration on lung morphology in CLP-induced sepsis.
(A) Representative Masson, PAS and H/E stained lung sections from Sham (I), CLP (II) and CLP + EPO (III) animals are shown. Original magnification × 400. CLP caused pulmonary injury as manifested by hyaline membranes (arrow); intra alveolar haemorrhages (star) and diffuse interstitial pneumonia (diamond). Note the inflammatory process with marked infiltration of leukocytes into interstitial and alveolar spaces, alveolar distortion, edema, and thickening of alveolar wall. Lung histopathologic damage was attenuated to a great extent by EPO administration.
(B) Scoring of lung injury. Scores were measured in Sham, CLP and CLP + EPO samples (n = 6 mice/each group). Values are mean ± SEM. *** $P < 0.001$ indicate significant differences.

which was mainly observed at the medullar interstitium and renal tubules.

Fig. 6B-II illustrates the outcomes of VEGF-R2 immunochemistry in lung samples. Likewise the renal tissue, the expression of this receptor was markedly decreased in CLP pulmonary samples compared to the sham group ($P < 0.001$). Conversely, EPO + CLP samples showed the enhancement of VEGF-R2 expression, which was mainly located in alveolar epithelium, predominantly in pneumonocytes Type II. Morphometric analyses of VEGFR-2 immunohistochemistry in both tissues are shown Fig. 6C.

4. Discussion

A complex network of events is set into motion in the body by the infection and results in the pathogenesis of sepsis. The endothelium is a major target of sepsis-induced events and endothelial cell damage accounts for much of the pathology of septic shock [18]. Endothelial dysfunction leads to an inability of the endothelial cells to maintain vascular tone with loss of blood pressure. In addition, endothelial damage leads to capillary leak with intravascular ume depletion and edema formation in inved organs. Moreover, it is well known that the severity of microvascular dysfunction correlates with the mortality of septic patients [19,20].

To the best of our knowledge, this is the first study that demonstrates the effect of exogenous EPO administration and the roles of EPO/EPO-R and VEGF/VEGF-R2 systems in the amelioration of kidney and lung acute injury during polymicrobial sepsis.

EPO treatment exerts complex actions for promoting the maintenance of homeostasis of the organism and for ameliorating the tissue-induced injury under diverse kinds of stress. It is well recognized that EPO rescues cells from apoptosis, reduces inflammatory responses, restores vascular autoregulation, and promotes healing.

Despite Takano et al. found that EPO, although effective in reducing lymphoid tissue apoptosis, is not associated with a survival benefit in the CLP model of experimental sepsis [20], this study revealed that EPO treatment determined a moderated short term survival advantage in agreement with Aoshiba's et al. report [15].

EPO exerts is biological functions through its receptor (EPO-R), which is extensively distributed in numerous tissues and organs, included vascular endothelial cells [21], kidney cells [22] bronchiolar epithelial cells, and type II alveolar epithelial cells [23].

The present results clearly show that the beneficial effects of EPO are mediated, in part, by the recovery of EPO-R, as evidenced by the higher levels of protein expression in kidneys and lungs of mice undergoing CLP-induced sepsis (Fig. 5).

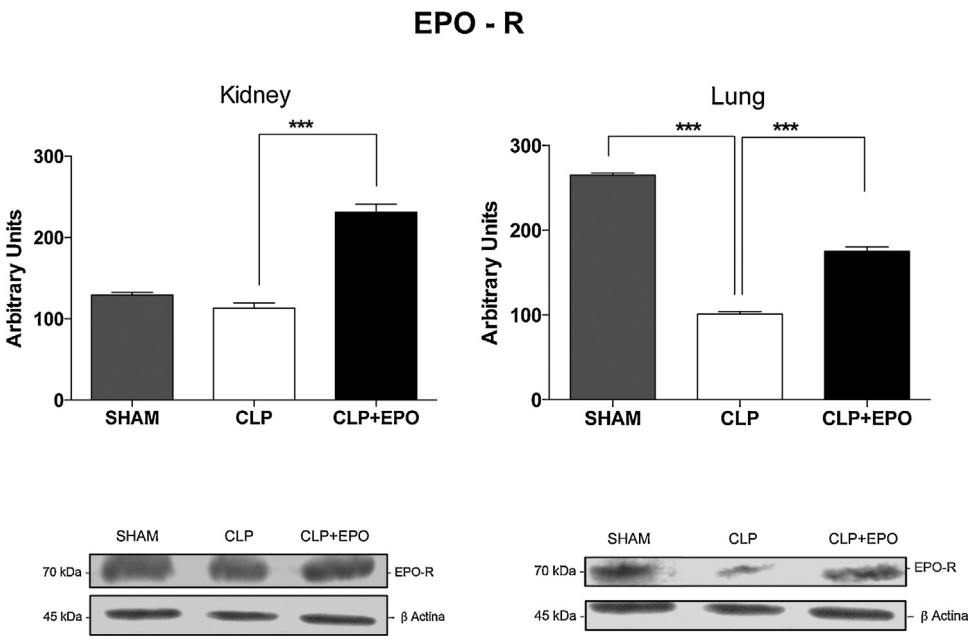

Fig. 5. Effect of EPO administration on EPO-R expression in kidneys and lungs during CLP-induced sepsis.
Representative blots of EPO-R (75 kDa) in renal and lung homogenates from Sham, CLP and CLP+ EPO samples (n = 6 mice/each group). Immunoblottings were performed by triplicate from a single sample. EPO administration induced the enhancement of EPO-R compared to CLP in both tissues. Data were normalized to β – actin used as loading control. Graphic bars represent densitometric arbitrary units of the ratio EPO-R/β actin. Values are mean ± SEM. *** $P < 0.001$ indicate significant differences.

AKI is common complication in septic patients and it is associated with renal hypoperfusion and microvascular dysfunction.

Recombinant human erythropoietin (rhEPO) administration was used for attenuating renal tubulointerstitial damage in several experimental settings of renal injury [16,24–26]. Moreover, a previous study of our research group has demonstrated that EPO exerts protective effects on an endotoxemic-related AKI model by the modulation of the intrinsic apoptotic pathway and the expression of EPO-R [27].

In the present study, the decrease of intratubular casts and renal tubular injury scores, as well as, the amelioration of renal histologic damage, clearly revealed the EPO protective effect in mice undergoing CLP. In addition, the EPO administration improved the renal function as evidenced by the reduction of BUN and creatinine levels. These results are in accordance with Mitra's et al. report [28] who used a LPS–induced sepsis and the outcomes from Aoshiba's et al. [15] study with both experimental models of septic shock.

An additional targeted organ for injury during sepsis is the lung, and the progressively impaired pulmonary function is the major complication of septic disease. The beneficial effects of EPO administration became evident with the significantly decrease of the lung injury scores compared to CLP samples (Fig. 4).

Once more, our results are in agreement with other studies that proved the beneficial effects of EPO on different experimental settings for ALI, such as, ischemia/reperfusion [29], LPS [30], zymosan induced-non septic shock [31], CLP [15] and hyperoxia [32].

We hypothesized that the EPO histoprotection on renal and pulmonary tissues in CLP septic model inves, in part, a pro-angiogenic effect that might be related to the increment of several key molecules as VEGF and VEGF-R2.

The biological properties of VEGF (angiogenesis, vascular permeability, endothelial survival, etc.) have been widely demonstrated in in vitro and in vivo systems. However, the relative contribution to tissue protection VEGF seem to be organ and context specific, and this biological property should be verified in each system [33,34].

In this study, experimental sepsis caused a significant reduction of the VEGF/VEGF-R2 pair expression in both, pulmonary and renal tissue.

Our results about renal VEGF decreased expression in septic conditions are in agreement with several reports [35,36]. However, there are controversial findings regarding VEGF-R2 occurrence in septic AKI [36,37].

On the other hand, our results related to the assessment of VEGF and VEGF-R2 in septic-induced ALI are in line with those reported by Tsokos et al. [38] and Jesmin et al. [39].

Although pulmonary occurrence of VEGF varies in different experimental settings of lung injury [40,41] and, there are limited studies concerning its expression in CLP-induced sepsis with exogenous EPO administration. Furthermore, the relationship between EPO treatment and the behaviour of VEGF/VEGF-R2 system in sepsis-induced –AKI is much less known.

Our data revealed that, in this experimental model, EPO mediates the attenuation in both tissues as supported by VEGF pulmonary over expression and the restoration in VEGF-R2 immunoreactivity (Fig. 6).

The outcomes of septic AKI and ALI of the present study are in agreement with di Villa Bianca et al. [42], who have demonstrated that EPO exerts a protective effect in LPS-induced septic shock by modulating endothelial dysfunction and vascular hyporeactivity in rats. Moreover, Nakano et al. [43] using a murine model of systemic ischemia, reported that EPO/EPO-R system plays an important role in angiogenesis through up regulation of VEGF/VEGF receptor pair, indicating that both systems are engaged.

Even though multiple mediators may induce VEGF production triggering capillary leak in sepsis; VEGF and EPO seem to be synergistic, or to be mutually inved in the context of sepsis induced –AKI and –ALI.

Taken together, our data suggest that the effect of exogenous EPO administration is related to the EPO/EPO-R and the VEGF/VEGF-R2 systems in the amelioration of kidney and lung acute injury during polymicrobial sepsis.

Our data is, however, limited to association and does not define regulation of VEGF/VEGF-R2 as the only mechanism of

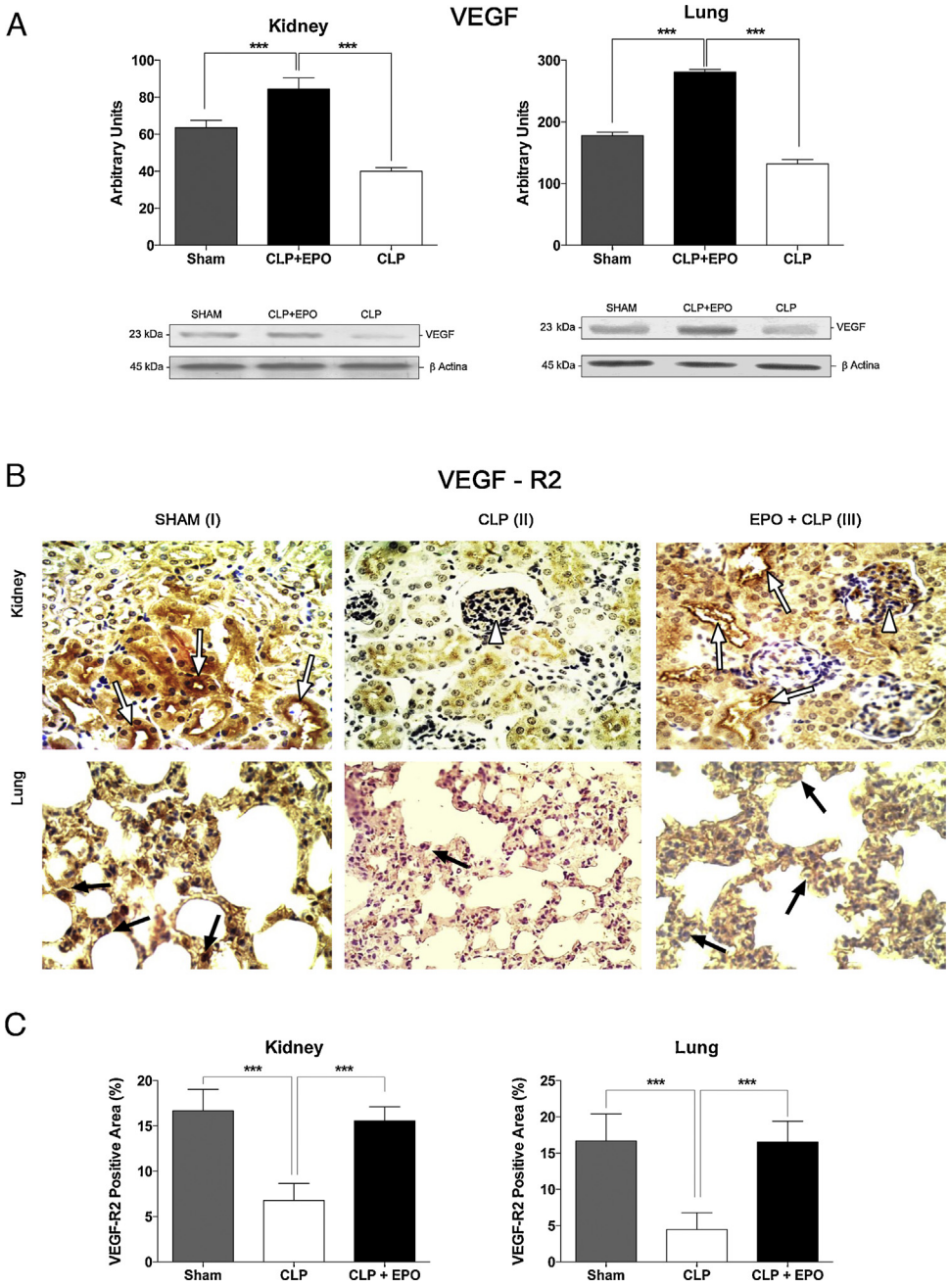

Fig. 6. Effect of EPO administration on the expression of VEGF and VEGF-R2 in kidneys and lungs during CLP-induced sepsis.
(A) Representative blots of VEGF (23 kDa) in renal and lung homogenates from Sham, CLP and CLP+ EPO samples (n = 6 mice/each group). Immunoblottings were performed by triplicate from a single sample. EPO administration induced the over expression of VEGF compared to CLP in both tissues. Data were normalized to β – actin used as loading control. Graphic bars represent densitometric arbitrary units of the ratio VEGF/β actin. Values are mean ± SEM. *** $P < 0.001$ indicate significant differences.
(B) Immunohistochemistry of VEGF-R2 in kidneys (I) and lung (II) sections from Sham, CLP and CLP+ EPO (n = 6 mice/each group). VEGF-R2 immunoreactions in renal sections (I) from Sham mice revealed that receptors were mainly located in tubular epithelial cells. The immunoreactivity exhibited a diffuse cytoplasmic pattern with strong apical VEGF-R2 expression (white arrows). Moreover, VEGF-R2 was noticed in the renal glomerular endothelial cells (arrow heads). CLP caused a remarkable reduction of VEGF-R2 immunoreactivity in both locations (tubular and glomerular endothelial cells), EPO treatment caused the restoration of VEG-R2 expression. The expression of VEGF-R2 in pulmonary sections (II) was confined to alveolar epithelial and endothelial cells. CLP caused a notable decrease of VEGF-R2 immunoreactivity in lungs. EPO administration induced the restoration of VEGF-2 expression in the alveolar epithelium, particularly in pneumonocytes type II (black arrows). Original magnification × 400.
(C) Semiquantitative evaluation of VEGFR-2 immunoreactive areas in Sham, CLP and CLP+ EPO (n = 6 mice/each group). Values are the mean percentages of VEGFR-2 positive area ± SEM. *** $P < 0.001$ indicates significant differences.

cytoprotection. The protective effects of EPO in lung and kidney septic induced-injury thus almost certainly include additional as yet undefined mechanisms. Further in vivo studies will be required to confirm these effects and to determine optimal timing and duration of therapy.

5. Conclusions

The present study demonstrates that the exogenous administration of EPO attenuated septic ALI and AKI. Moreover, we propose that the protective effect of EPO against renal and pulmonary

injury during sepsis may be in part, due to its pro-angiogenic effect through the over expression of the VEGF/VEGF-R2. Thus, it would be a functional relationship between the EPO/EPO-R and VEGF/VEGF-R2 systems in promoting vascular and epithelial cells response against septic injury. Our findings support the potential use of EPO rh or its analogues, as therapeutic agents for sepsis-induced ALI and AKI.

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