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K Kannan

Division of Pharmacology and Toxicology, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India

S Choudhury

Division of Pharmacology and Toxicology, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India

T Ramasamy

Assistant Professor, Department of Veterinary Pharmacology and Toxicology Madras Veterinary College, Vepery, TANUVAS, Chennai, Tamil Nadu, India

M Kesavan

Division of Pharmacology and Toxicology, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India

S Parida

Division of Pharmacology and Toxicology, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India

TU Singh

Division of Pharmacology and Toxicology, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India

Corresponding Author:**T Ramasamy**

Assistant Professor, Department of Veterinary Pharmacology and Toxicology Madras Veterinary College, Vepery, TANUVAS, Chennai, Tamil Nadu, India

Erythropoietin prevents liver injury in septic mice

K Kannan, S Choudhury, T Ramasamy, M Kesavan, S Parida and TU Singh

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Abstract

In the present study, the effect of erythropoietin in liver of septic mice was investigated. Sepsis was induced by caecal ligation and puncture model. After 20+2 hrs of surgery, blood and liver samples were collected. Plasma TNF- α , IL-1 β , Serum AST, ALT and Liver lipid peroxidation, myeloperoxidase, thiols was estimated and histopathology was performed. Post-treatment with erythropoietin ameliorated the sepsis induced changes in hepatic and systemic inflammatory changes in mice.

Keywords: Erythropoietin, sepsis, mice, liver

Introduction

Sepsis is dysregulated host response to infection and is characterised by life-threatening organ dysfunction^[1]. Recent studies have revealed that liver has a vital role in sepsis, and is involved in the regulation of immune defence during systemic infections by mechanisms such as bacterial clearance, acute-phase protein or cytokine production and metabolic adaptation to inflammation. In addition, activated neutrophils, which are also recruited to the liver and produce potentially destructive enzymes and oxygen-free radicals, may further enhance acute liver injury. Hepatic failure is traditionally considered as a late manifestation of sepsis-induced multiple organ dysfunction syndromes^[2]. Erythropoietin (EPO), a 31-kDa glycoprotein produced by fetal liver and adult kidneys and its receptor is expressed in the brain, heart, lungs, liver, kidneys, vascular endothelium, and lymphoid tissues, and upregulated by hypoxic stimulation. Recent studies demonstrated cardioprotective effect of erythropoietin in a CLP model of sepsis in rats through improvement in left ventricular functions^[3]. Similarly EPO reverses sepsis-induced vasoplegia to Noradrenaline through the preservation of α 1D adrenoceptor mRNA/protein expression; inhibition of GRK2 mediated desensitization and attenuation of NO overproduction in the mouse aorta^[4]. Therefore the present study was undertaken to investigate the effect of erythropoietin post-treatment on sepsis-induced hepatic and systemic inflammatory changes in mice model of sepsis.

Materials and Methods**Experimental Animals**

Healthy male Swiss Albino mice (30-35 g) were procured from the Laboratory Animal Resource Section, Indian Veterinary Research Institute, Izatnagar, and Uttar Pradesh, India. Mice were housed in different polypropylene cages with free access to feed and water in the Divisional animal house.

Induction of sepsis in mice

Caecal ligation and puncture was produced as described⁵. Mice were fasted overnight before the induction of sepsis, but allowed water *ad libitum*. The animals were anesthetized by i.p. injection of xylazine (10 μ g/g body wt.) and ketamine (80 μ g/g body wt.). A 2-cm ventral midline incision was performed. Then the caecum was exposed and ligated with 3/0 silk just distal to the ileocecal valve to avoid intestinal obstruction, punctured twice with a 21 G needle and returned to the abdomen. The abdominal incision was closed in layers. Normal saline (1 mL/mouse) was given subcutaneously to all mice to prevent dehydration. Sham-operated mice

had undergone the same surgical procedure except cecal ligation and puncture. All the surgical maneuvers were carried out according to the procedures laid down by the Institutional Animal Ethics Committee.

Drug administration

Erythropoietin was procured from Zuventus healthcare Ltd, a joint Venture of Emcure®, Mumbai. SO / CLP mice were injected subcutaneously with normal saline (0.1 ml) and SO+EPO/CLP+EPO mice were injected erythropoietin (1000 IU/kg) subcutaneously. The dose and time of administration of erythropoietin was based on previous reports [6, 7]

Collection of blood and liver

After 20±2 h surgery, the mice (sham operated/sepsis) were anaesthetized with xylazine and ketamine intraperitoneally, and blood was collected in tubes (BD vacutainer® 3.5 cc) coated with clot activator. Plasma was separated by centrifuging the blood at 4000 rpm for 10 min at 4 °C. The samples were then immediately aliquoted and stored at -80 °C for nor-adrenaline (NA) measurement. Liver was removed, cleaned and stored at -80 °C for further studies.

Parameters studied

Assay of inflammatory cytokines in plasma

Assessment of inflammatory cytokines like IL-1 β and TNF- α level in plasma from different groups was done using commercially available ELISA kits (Cloud-Clone Corp, USA) by following manufacturer's protocol.

Plasma nitrite [(NO)x] measurement

Nitrite was estimated based on the procedure explained⁸

Serum AST/ALT

Serum AST and ALT levels were assayed using the kits of Span Diagnostics (India) as per the manufacturer's instructions.

Measurement of lipid peroxidation (LPO) in liver

The extent of lipid peroxidation was evaluated in terms of MDA (malondialdehyde) production determined by the thiobarbituric acid (TBA) method [9].

Assay of myeloperoxidase activity in liver

Neutrophil infiltration was assessed in terms of myeloperoxidase activity (MPO) in liver. After 20±2 hrs of sepsis mice were anesthetized by injection of pentobarbitone sodium (60 mg/kg body wt. *i.p.*). The liver were cut, weighed and homogenized in 0.5% CTAB buffer (CTAB in 50 mM potassium phosphate buffer) to make 10% homogenate. After centrifugation at 15,000 rpm for 2 min, supernatant fractions were assayed for MPO activity [10]. Supernatant (0.1 mL) was added to 2.9 mL of 50 mM potassium buffer (pH 6.0) containing 0.167mg/mL o-dianisidine dihydrochloride and 0.001% hydrogen peroxide. The sample absorbance was measured at 460 nm for 2 min. The molar extinction coefficient for o-dianisidine at 460nm was taken as 1.13x10⁴ M⁻¹.cm⁻¹ and one unit MPO activity is defined as amount of enzyme that reduces 1 μ mol of peroxide/min. The MPO activity is expressed as unit/g tissue.

Liver nitrate measurement

Liver nitrate was estimated as per the method described earlier with some modifications [11].

Determination of Total Thiols (T-SH)

Total sulfhydryl groups were determined by measuring the colour complex formed by the reaction of sulfhydryl groups with diethylnitrobenzoic acid (DTNB), as per the procedure [12].

Histological examination

The liver samples of mice from different groups were fixed in normal 10% buffered formalin for 48 h, dehydrated in ascended alcohol series and embedded in paraffin wax. Approximately 6- μ m-thick sections were stained with hematoxylin and eosin (H&E) for the assessment of the general morphology with light microscopy [13].

Results and Discussion

Systemic anti-inflammatory effects of erythropoietin in sepsis

Sepsis is often associated with an increase in pro-inflammatory cytokines and as a marker of sepsis, inflammatory cytokines such as IL-1 β and TNF- α were measured in plasma of mice from different groups. As shown in Table 1, sepsis significantly increased the plasma level of IL-1 β to 6327±625.40 pg/mL (n=6) compared to that of SO mice (156.9±32.72 pg/mL, n=6). Treatment with EPO significantly reduced the IL-1 β level to 3531.00±927.80 pg/mL n=6, in plasma from septic mice. However, erythropoietin treatment did not produce any significant change in the level of IL-1 β in SO mice compared to that of SO mice treated with the vehicle.

TNF- α level in plasma of SO mice was 208.40±19.63 pg/mL (n=6). As shown in Table 1 sepsis significantly increased the level to 1208.00±153.20 pg/mL (n=6). Treatment with EPO significantly reduced the TNF- α level to 619.90±131.30 pg/mL (n=6) in septic mice. However, treatment with EPO in SO mice did not produce any significant alteration in plasma TNF- α level compared to vehicle-treated SO mice.

The pro-inflammatory cytokines involved in the compromise of endothelial integrity during sepsis include TNF- α , which induces disruption of the lung endothelium in patients leading to increase in permeability to protein [14]. Sheep injected with recombinant human TNF- α displayed a septic-like state, whereas antibodies against TNF- α reduced mortality in primate models of sepsis [15]. Previous reports from rat splanchnic artery occlusion shock model had shown plasma TNF- α concentrations decreased with erythropoietin treatment [16]. Similarly, erythropoietin reduced the TNF- α concentrations in the kidney of CLP rats [17]. Based on the findings that erythropoietin decreased pro-inflammatory cytokines (TNF- α and IL- β) levels in plasma indicate that overall systemic inflammatory response was reduced with erythropoietin post-treatment in sepsis.

Plasma nitrite level

As shown in Table 1, sepsis caused significant increase in the plasma nitrite level (17.49±0.87 nmol/mL, n=6) compared to SO mice (4.83±0.33 nmol/mL, n=7). Treatment with EPO significantly reduced the plasma nitrite level to 9.57±1.10 nmol/mL (n=6) in septic mice. However, SO mice treated with EPO not shown significant difference in plasma nitrite level compared to SO mice.

Similar to vascular tissue, we observed that erythropoietin prevented overproduction of iNOS-derived NO in plasma. This finding in the CLP model of sepsis is consistent with the findings of Kathirvel and co-workers¹⁸, who demonstrated increased plasma nitrite level in mice model of sepsis. It is

well known that cytokines are inducers of iNOS. We found that sepsis increased the plasma pro-inflammatory cytokines such as TNF- α and IL- β and erythropoietin post-treatment significantly attenuated the rise in cytokine levels. This could be the probable mechanism how erythropoietin was able to decrease plasma nitrite concentrations.

Effect of erythropoietin post-treatment in sepsis induced liver injury in mouse

Serum AST and ALT

Sepsis induced liver injury is associated with an increase in AST and ALT and as a hepatic marker of sepsis; AST and ALT were measured in serum of mice from different groups. As shown in Table 2, sepsis significantly increased the serum AST to 157.8 ± 10.67 IU/L (n=6) compared to that of SO + vehicle treated mice (71.26 ± 2.89 IU/L, n=6). Treatment with EPO significantly reduced the serum AST level to 125.9 ± 11.38 IU/L n=6, in plasma from septic mice. However, erythropoietin treatment did not produce any significant change in the level of AST in SO mice compared to that of SO mice treated with the vehicle.

ALT level in serum of SO + vehicle treated mice was 22.52 ± 1.49 IU/L (n=6). As shown in Table 2, sepsis significantly increased the level to 80.4 ± 5.46 IU/L (n=6). Treatment with EPO significantly reduced the serum ALT level to 56.8 ± 6.96 IU/L (n=6) in septic mice. However, treatment with EPO in SO mice did not produce any significant alteration in serum ALT level compared to vehicle-treated SO mice.

Increased activities of serum ALT and AST are known to be the indicators of hepatic damage. In a CLP model of mice, a rise in plasma levels of AST and ALT was observed [19] which was attributed to the loss of structural integrity of hepatocytes. Similarly, increased levels of AST and ALT were observed in patients dying from sepsis [20]. As observed in the present study, erythropoietin post treatment was able to reverse the rise in serum AST and ALT levels which is in accordance with the decrease in serum ALT levels of erythropoietin-treated endotoxaemic mice [6].

Septic shock, a severe form of sepsis responsible of high mortality rates in intensive care units, is associated with the development of progressive damage to multiple organs [21, 22]. Among the affected organs, dysfunction of the liver is an important indicator of poor outcome [23]. During sepsis, microorganisms housed in the alimentary tract invade the blood stream and release various substances, which activate the endogenous mediators of the host systemic response. This activation leads to the release of massive amounts of oxidants, proteases, arachidonic acid metabolites, which further trigger local and systemic inflammatory responses in remote organs [24]. Both clinical and experimental studies have shown that any noxious tissue event, including sepsis, is perceived by macrophages and monocytes, which in turn secrete cytokines such as interleukin-1 (IL-1) and TNF- α [25, 26]. As evidenced in the present study, sepsis induction resulted in increased TNF- α , indicating the role of this cytokine in sepsis-induced systemic inflammation. On the other hand, erythropoietin post treatment attenuated the plasma TNF- α response in sepsis. It was previously shown that erythropoietin pre-treatment reduces the TNF- α in kidney of CLP rats [18]. Similarly increased plasma IL-1 β level observed in the present study was restored in the EPO-treated septic mice. Thus, it seems likely that the amelioration of sepsis-related organ failure by erythropoietin involves the suppression of a variety of pro-inflammatory mediators produced by the leukocytes and macrophages. The pro-inflammatory cytokines activate

inflammatory cells such as neutrophils, macrophages or monocytes, platelets, and mastocytes, which release large amounts of the toxic oxidizing ROMs, which cause cellular injury via several mechanisms including the peroxidation of membrane lipids, and the oxidative damage of proteins and DNA [27].

Liver LPO

Table 2 summarize the effect of erythropoietin post-treatment on lipid peroxidation in liver of septic mice. Sepsis significantly increased the LPO level to 90.96 ± 8.02 nmol MDA/g tissue (n=6). However, erythropoietin post-treatment significantly reduced LPO level to 61.87 ± 7.85 nmol MDA/g tissue (n=6) in septic mice. Erythropoietin treatment in SO mice did not cause any significant change in lipid peroxidation compared to vehicle-treated SO mice. Many reports suggest that reactive oxygen metabolites (ROM) and ROM-induced lipid peroxidation play important roles in the pathogenesis of sepsis and its complications [28, 29]. Lipid peroxidation can cause changes in membrane fluidity and permeability, which will eventually lead to cell lysis [30]. In the present study, the elevated levels of MDA, an end product of lipid peroxidation, in the liver were suppressed by erythropoietin, indicating that erythropoietin reduces lipid peroxidation and thereby supports the maintenance of cellular integrity. In many diseases and acute inflammatory disorders, important components of the pathological processes are linked to the ability of neutrophils to release a complex assortment of agents that can destroy normal cells and dissolve connective tissues [31]. Evidence suggests that neutrophils release chemotactic substances, such as interleukin 8, which promotes neutrophil migration to the tissue, activates neutrophils, and increases the damage [32, 33]. Previous reports suggest that ROMs play a role in the recruitment of neutrophils into injured tissues, but activated neutrophils are also a potential source of ROMs [34]. ROMs can generate hypochlorous acid (HOCl) in the presence of neutrophil-derived MPO, and initiate the deactivation of antiproteases and activation of latent proteases, which cause tissue damage [35]. In the present study, the presence of elevated MPO activity in hepatic tissue and the increased level of plasma TNF- α indicate the contribution of PMN infiltration and the impact of pro-inflammatory TNF- α in sepsis-induced tissue damage. Since erythropoietin post treatment inhibited sepsis-induced increase in MPO activity and abolished the TNF- α response concomitantly, our results suggest that neutrophil accumulation contributes to sepsis-induced oxidative injury and the protective effect of erythropoietin, in part, is dependent on its inhibitory effect on tissue neutrophil infiltration and neutrophil-associated TNF- α response.

Liver Thiols

Liver total thiols ($\mu\text{mol/g}$ tissue) have been presented in Table 2. Sepsis significantly decreased the total thiols to 0.34 ± 0.04 $\mu\text{mol/g}$ tissue (n=6). However, erythropoietin post-treatment significantly increased liver total thiols to 0.48 ± 0.039 $\mu\text{mol/g}$ tissue (n=6) in septic mice. Erythropoietin treatment in SO mice did not cause any change in total thiols compared to SO+vehicle group (0.55 ± 0.04 $\mu\text{mol/g}$ tissue, n=6). Among all the antioxidants that are available in the body, thiols constitute the major portion of the total body antioxidant defense against ROS. T-SH plays a major role along with other antioxidants in the body to ameliorate the lipid peroxidative effects of ROS [36]. In the present study a

significant decrease in T-SH content in liver was observed suggesting decreased oxygen radical scavenger activity in sepsis and post-treatment with erythropoietin restored T-SH levels indicating reversal of CLP-induced thiols depletion and exerting the antioxidant effect.

Liver Myeloperoxidase

Table 2 present the effect of erythropoietin post-treatment on myeloperoxidase activity in liver of septic mice. Sepsis significantly increased the MPO activity to 7.63 ± 1.08 units/mg tissue (n=6) compared to SO mice (2.48 ± 0.44 units/mg tissue, n=6). Treatment with erythropoietin significantly reduced the MPO activity to 4.43 ± 1.20 units/mg tissue, (n=6). In SO+EPO mice, liver myeloperoxidase activity was 3.06 ± 0.88 units/mg tissue (n=6) which was comparable to SO mice.

eNOS derived nitrate content in mouse liver

Table 3 summarize the effect of different treatments in sepsis on constitutive total nitrate level in mouse liver. The constitutive nitrate content in liver from SO animals was 4.05 ± 0.24 pmol/mg tissue (n=6). Sepsis significantly decreased the constitutive nitrate content to 2.45 ± 0.53 pmol/mg tissue (n=6). Treatment with EPO significantly increased the nitrate level to 3.84 ± 0.50 pmol/mg tissue (n=6). In SO mice erythropoietin did not cause any change in constitutive nitrate content (4.35 ± 0.27 pmol/mg tissue, n=6) compared to vehicle-treated SO animals.

Inducible Nitrate content in mouse liver

Table 3 summarize the effect of different treatments on inducible nitrate content in mouse aorta. iNOS-derived nitrate level in liver from SO animals was 0.19 ± 0.05 pmol/mg tissue (n=6). Sepsis significantly increased aortic nitrate level to 2.99 ± 0.42 pmol/mg tissue (n=6). Treatment with EPO in septic mice significantly reduced the nitrate level (1.41 ± 0.41 pmol/mg tissue, n=6). Erythropoietin in SO mice did not significantly alter the iNOS-derived nitrate level in aorta compared to SO.

Histopathological changes in mouse liver

After 20 ± 2 hrs after surgery, the liver biopsies from all the treatment groups were processed for assessment of tissue damage by histopathological examination. The histopathological appearance of the liver in SO mice was normal as shown in H&E-stained sections of liver (Fig.1). In septic mice, livers were congested macroscopically and the changes recorded in histological observations were mainly severe infiltration of polymorphonuclear granulocytes, numerous vacuoles in hepatocytes suggestive of vacuolar to hydrophic degeneration (Fig.2). However, liver preparation from SO+EPO mice showed some infiltration of polymorphonuclear granulocytes (PMN) (Fig.3). Treatment of SO mice with erythropoietin showed normal appearance in septic mice (Fig 4). Sepsis causes an increase in inflammation characterized by extensive neutrophil infiltration as a result of reduced vascular barrier and degenerative changes. Earlier reports [37] demonstrated that marked inflammation in response to CLP was characterized by congestion, numerous focal necroses of hepatocytes, kupffer cell hyperplasia and bile duct proliferation in septic liver of rat. Similarly, in mice model of endotoxaemia, hepatocyte necrosis, neutrophil infiltration and swollen and vacuolated hepatic cells were reported [6]. In the present study histo-pathological examination revealed that erythropoietin post-treatment reduced neutrophil infiltration and degeneration was not pronounced in liver. The biochemical findings corroborate the histopathological changes in liver in sepsis and their amelioration by erythropoietin treatment.

In conclusion, the present study demonstrates that erythropoietin post treatment alleviated sepsis-induced impairment in liver functions which was evident from reduction in sepsis-induced elevations of serum biomarkers, such as AST/ALT activity, and pro-inflammatory cytokines (TNF- α /IL-1 β levels). Furthermore, erythropoietin treatment reversed increased lipid peroxidation, MPO activity and iNOS mediated NO production along with decreased T-SH levels in the liver following sepsis.

Table 1: Effect of erythropoietin post-treatment on Plasma nitrite level (nmol/mL), Plasma IL-1 β (pg/mL), Plasma TNF- α (pg/mL) in septic mice

Groups(n=6)	Plasma nitrite (nmol/mL)	IL-1 β (pg/mL)	TNF- α (pg/mL)
SO+ vehicle	4.83 ± 0.33	156.9 ± 32.72	208.4 ± 19.63
CLP+vehicle	$17.49 \pm 0.87^*$	$6327 \pm 625.4^*$	$1208 \pm 153.2^*$
SO+EPO	6.49 ± 0.38	318.6 ± 175.7	247 ± 34.78
CLP+EPO	$9.57 \pm 1.10^\#$	$3531 \pm 927.8^\#$	$619.9 \pm 131.3^\#$

Results are expressed as Mean \pm SEM

* $p < 0.001$ compared to SO+vehicle, # $p < 0.001$ compared to CLP+vehicle

Table 2: Effect of erythropoietin post-treatment on serum ALT (IU/L), serum AST (IU/L), liver LPO, Liver Total thiols and Liver MPO in septic mice

Groups (n=6)	ALT (IU/L)	AST (IU/L)	Liver LPO (nmol/g)	Liver Total thiols (μ mol/g)	Liver MPO (units/g)
SO+ vehicle	22.52 ± 1.49	71.26 ± 2.89	57.82 ± 4.2	0.55 ± 0.05	2.48 ± 0.45
CLP+vehicle	$80.4 \pm 5.46^{**}$	$157.8 \pm 10.67^{**}$	$90.96 \pm 8.02^\$$	$0.34 \pm 0.04^\$$	7.63 ± 1.08^{SS}
SO+EPO	31.03 ± 3.65	87.09 ± 4.44	65.38 ± 7.90	0.52 ± 0.06	3.06 ± 0.89
CLP+EPO	$56.8 \pm 6.96^{##}$	$125.9 \pm 11.38^\#$	$61.87 \pm 7.84^\#$	$0.48 \pm 0.03^\#$	$4.43 \pm 1.15^\#$

Results are expressed as Mean \pm SEM

** $p < 0.001$ in comparison to SO+Vehicle, ## $p < 0.01$ in comparison to CLP+Vehicle

^\\$ $p < 0.05$ in comparison to CLP+Vehicle, \\$ $p < 0.05$ in comparison to SO+Vehicle

SS $p < 0.01$ in comparison to SO+Vehicle

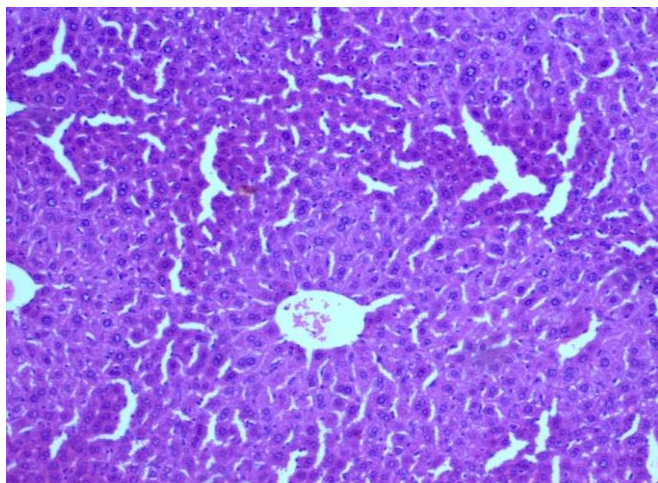
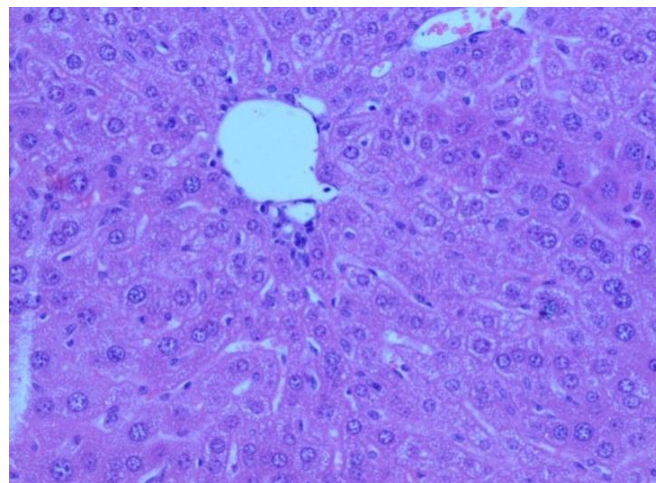
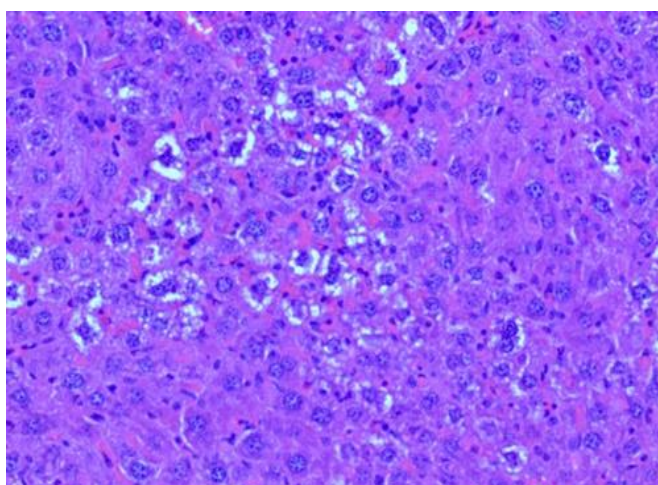
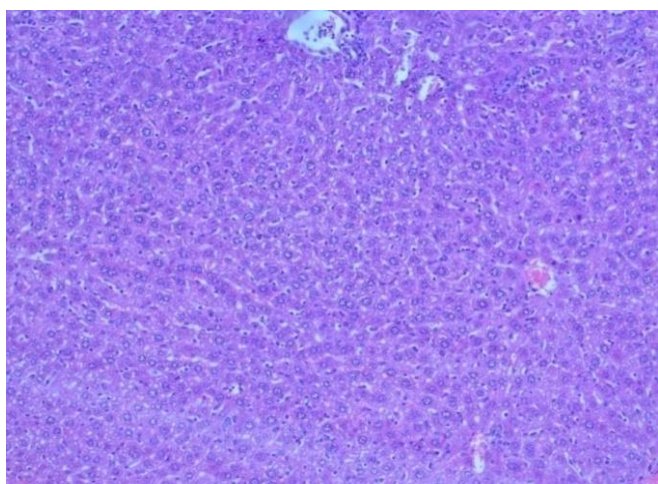
Table 3: Effect of erythropoietin post-treatment on eNOS and iNOS derived nitrate content in liver of septic mice

Groups (n=6)	eNOS derived nitrate content (pmol/mL homogenate)	iNOS derived nitrate content (pmol/mL homogenate)
SO+ vehicle	4.04±0.247	0.193±0.05
CLP+vehicle	2.45±0.53*	2.99±0.41**
SO+EPO	4.35±0.28	0.31±0.07
CLP+EPO	3.84±0.50#	1.41±0.42###

Results are expressed as Mean±SEM

* $p < 0.05$ in comparison to SO+Vehicle, # $p < 0.05$ in comparison to CLP+Vehicle

** $p < 0.001$ in comparison to SO+Vehicle, ### $p < 0.01$ in comparison to CLP+Vehicle

**Fig 1:** Microscopic view of Liver (SO+vehicle)**Fig 4:** Microscopic view of Liver (CLP+EPO)**Fig 2:** Microscopic view of Liver (CLP+vehicle)**Fig 3:** Microscopic view of Liver (SO+EPO)

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