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Caecal Ligation and Puncture Develops Time Dependent Progression of Sepsis with Multiple Organs Damage and Vascular Dysfunctions in Mice

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Abstract

Sepsis is a dysregulated systemic inflammatory response syndrome that affects multiple organs. However, its effect on vital organs during different phases of sepsis is lacking. Present study was carried out to establish the time dependent changes in the vital organs during different phases of sepsis. Sepsis was induced by caecal ligation and puncture in mice. Sepsis significantly reduced RBC, Hb and WBC counts during both the phases whereas neutrophil count was increased during early phase. There was also a marked fall in lymphocyte count during late phase of sepsis which is an indicative of immunosuppressive state. Significant rise in the plasma ALT, AST, BUN and creatinine levels during early and late phases of sepsis were suggestive of liver and kidney dysfunctions which were further substantiated by histopathological examinations of these vital organs. Sepsis also produced a state of hypoproteinaemia with significant reduction in plasma albumin level. Significant progressive attenuation of vascular reactivity to nor-adrenaline and endothelial relaxation to acetylcholine were also observed in early to late phases of sepsis. However, sodium-nitroprusside-induced endothelium-independent relaxation was unaltered in both early 'as well as late phase of sepsis. Histopathological examination of lungs, heart and intestine showed progressive degenerative changes which were more prominent with progression from early to late phase of sepsis. Based on the findings of the present study, it may be inferred that caecal ligation and puncture produces time-dependent progression of sepsis in mice affecting multiple organs.

Keywords: CLP, Early and Late Phase, Histopathology, Sepsis, Vital Organs

1. Introduction

Sepsis is a multifactorial disease with uncontrolled systemic production of inflammatory mediators ('cytokine storm') leading to Systemic Inflammatory Response Syndrome (SIRS) following microbial infection[1]. It is characterized by vascular hyporeactivity, decreased tissue perfusion, tachypnoea, hyperthermia, systemic hypotension, multiorgan failure despite antibiotic therapy, adequate fluid resuscitation and administration of vasopressors[2]. Infections caused by bacteria (Gram +ve or Gram -ve), fungi, virus, parasites, etc. or pathological conditions like ischemia, burn, abdominal surgery, pancreatitis,

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haemorrhage etc. favour the development of niche for sepsis. In United States, sepsis ranks tenth leading cause of death with mortality rates varying between 30 to 70% among ICU patients[3].

Caecal Ligation and Puncture (CLP) model leads to peritoneal contamination with mixed flora in the presence of devitalized tissue and thus bears an obvious large simulation to clinical syndrome in sepsis. The early hyperdynamic phase (hyperinflammatory) is characterized by increased vascular permeability, increased cardiac output, decreased systemic vascular resistance, decreased tissue perfusion, normal stroke volume and high heart rate[4]. The late hypodynamic phase (immunosuppressive) occurs due to myocardial dysfunction and is associated with reduced cardiac output, elevated systemic vascular resistance, hypotension, pulmonary hypertension, myocardial depression, heart failure, refractoriness to vasoconstrictive agents and finally death[5]. Although it is well reported that sepsis causes multiple organ dysfunctions, the systematic study elucidating the different phases of sepsis on the functions of vital organs is lacking. In the present study, we have reported that time dependent effect of sepsis on histo-pathological architecture of different vital organs along with the hemato-biochemical parameters.

2. Material and Methods

2.1 Experimental Animals

Healthy adult male Swiss albino mice (30–35 g) were procured from the Disease Free Small Animal House, Lala Lajpat Rai University of Veterinary and Animal Sciences (LUVAS), Hisar, Haryana, India. The animals were kept in Departmental Laboratory Animal House with proper managemental conditions of constant room temperature (23°C), humidity (50%) and 12-12 h light-dark cycle. Mice were housed in polypropylene cages with free access to feed and water throughout the study period. Animals were divided into three groups (*viz*. Gr-I: Sham-operated control (SO); Gr-II: Early phase of sepsis (CLP-6h) and Gr-III: Late phase of sepsis (CLP-20h)) with six animals in each group. All the experimental protocols were carried out after the approval from the Institutional Animal Ethics Committee (IAEC) (Approval No.: 39/02/2016 dated 16.09.2016).

2.2 Induction of Sepsis in Mice

Sepsis was induced by Caecal Ligation and Puncture (CLP) as described earlier[6]. This animal model of sepsis replicates the clinical sepsis observed in humans. Briefly, mice were fasted overnight before surgery but allowed water *ad libitum*. The animals were anesthetized using xylazine (10 µg/g body weight, *i.p*) and ketamine (80 µg/g body weight, *i.p*) and 2 cm ventral midline abdominal incision was made. Caecum was mobilized, ligated with 3-0 silk just distal to the ileocaecal valve to avoid the intestinal obstruction and then punctured into two locations with a 21G needle, allowing release of feces pursuing to peritonitis. The abdominal incision was closed with sterile sutures in layers after repositioning of caecum. To prevent dehydration, isotonic sodium chloride solution (1 ml per mouse) was injected subcutaneously to all the mice. Sham-operated (SO) mice were subjected to the same surgical procedure, except caecal ligation and puncture. All the surgical maneuvers were carried out according to the procedures laid down by the Institutional Animal Ethics Committee.

2.3 Quantification of Haematological and Blood Biochemical Parameters

Blood was collected through cardiac puncture at 6 h and 20 h after CLP in EDTA containing vials and haematological parameters were estimated. Plasma samples were collected by centrifuging the blood at 4000 rpm for 10 min and the blood biochemical analytes, namely ALT, AST, BUN, creatinine, albumin and total proteins, were estimated using commercially available kits (Span Diagnostics, India) with the help of semi-auto analyzer (Erba Manheim).

2.4 Isometric Tension Recording and Experimental Protocol

At 6 h and 20 h post-CLP, mice were sacrificed by bleeding from vena cava under xylazine–ketamine anesthesia. The lung and heart were taken out en-bloc after opening the chest and abdomen and the thoracic aorta was immediately placed into cold (4°C) modified Krebs–Henseleit solution (MKHS) with the following composition (mmol/l): 118.0 NaCl, 4.7 KCl, 2.5 $CaCl_2$,$2H_2O$, 1.2$MgSO_4$, $7H_2O$, 1.2 KH_2PO_4, 11.9 $NaHCO_3$, and 11.1 d-glucose. Thoracic aorta was cleaned off adhering tissue and cut into 3 to 4 mm long rings.

These arterial rings were mounted between two 'L' shaped hooks made from 37 G stainless steel wire and kept under a resting tension of 1.0 g in a thermostatically controlled (37.0 ± 0.1°C) organ bath (Ugo Basile, Italy) of 10 ml capacity containing MKHS and were continuously bubbled with medical gas (74% N_2+21%O_2+5% CO_2). The arterial rings were equilibrated for 60-80 min in organ bath filled with MKHS before recording tension. During equilibration period, the bath fluid was repeatedly changed once in every 15 min. The change in tension was measured by a high-sensitivity isometric force transducer and recorded using Lab Chart V6.1.3 Pro software program (Powerlab;

AD Instruments, Australia). After equilibration, arterial rings were contracted with high K+ (80 mM) depolarizing solution to assess the viability of the tissue. The preparations were washed with normal MKHS to restore the baseline resting tension. After a lapse of 30 min and 2 to 3 washes with normal MKHS, the concentration-dependent (0.1 nM – 1 µM) contractile response to noradrenaline (NA) was elicited in the arterial rings from different groups at an increment of 0.5 log unit. In order to evaluate the aortic response to vasodilator drugs (acetylcholine and sodium nitroprusside), Matching pre-contractions were elicited using phenylephrine at concentrations of 1 or 3 µM in control and sepsis groups, respectively. Different concentrations of phenylephrine were used because sepsis reduced the maximal response to vasoconstrictor drugs.

2.5 Histopathological Analysis

Tissue samples comprising of liver, kidney, spleen, lung, heart and intestine of mice at early (6 h post-surgery) and late (20 h post-surgery) phases of sepsis and sham-operated were harvested, fixed in 10% buffered formal-saline for 48 h, dehydrated in ascending alcohol grades and embedded in paraffin wax. Approximately 5–6 µm thick sections were prepared and stained with hematoxylin and eosin (H&E) for assessment of the general morphology under light microscope (Motic, China).

2.6 Data Analysis

Data were analyzed using Graph Pad Prism version 4.00 (San Diego, California, USA). Results are expressed as mean ± SEM with 'n' equals to number of animals used in the experiment.

The E_{max} (the maximum contractile response), R_{max} (the maximum relaxant response) and EC_{50} (the concentration producing 50% of the maximal response) were determined by nonlinear regression analysis (sigmoidal dose–response with variable slope) using GraphPad Prism version 4.00 (San Diego, California). Sensitivity/potency was expressed as $pD_2 = -\log EC_{50}$. Data related to concentration-dependent agonist response were analyzed by two-way analysis of variance (ANOVA) followed by Bonferroni *post-hoc* test. For comparing single variable between groups, one-way ANOVA followed by Newman-Keuls *post-hoc* analysis was applied to determine statistical significance. Differences in the mean values from different groups were considered statistically significant at $p<0.05$.

3. Results

3.1 Effect on Haematological Parameters

The effect of sepsis on haematological parameters of mice during early (CLP-6h) and late (CLP-20h) phases are presented in Table 1. Sepsis significantly ($p<0.05$) decreased the total erythrocyte (RBC) count and haemoglobin (Hb) levels during both the early and late phases as compared to that in SO mice. Total leukocyte count (WBC) during late phase of sepsis was significantly ($p<0.05$) reduced as compared to that in SO mice. In early phase of sepsis too, the WBC count was reduced by 28.37 % as compared to SO mice, however, the fall in WBC count was not statistically significant.

Differential leukocyte counts of cells revealed that there was significant ($p<0.05$) fall in lymphocytes during late phases of sepsis as compared to SO mice. However, the neutrophils count was significantly ($p<0.05$) increased during the early phases while decreased in the late phase of sepsis. The difference in the neutrophil count between the early and late phases of sepsis was also found to be statistically significant ($p<0.05$). No significant change in the monocytes count was observed during the different phases of sepsis in mice.

Table 1. Effect of different phases of sepsis on hematological profiles in mice

Group (n=6)	RBC (x106/µl)	Hb (g/dl)	WBC (x103/µl)	Lymphocyte (%)	Neutrophil (%)	Monocyte (%)
SO	9.31 ± 0.42	13.06± 0.56	7.40±1.09	80.67 ± 1.87	17.83 ± 1.74	0.8 ± 0.37
CLP-6h	3.08 ± 0.42*	10.97±0.45*	5.30±1.19	75.25 ± 1.47	30.50 ± 2.96*	2.50 ± 1.19
CLP-20h	4.73 ± 0.84*	10.25±0.66*	3.13±0.55*	67.00 ±1.35*#	14.00 ± 4.18#	2.00 ± 1.41

Data are presented as mean ± SEM, n=6. *$p<0.05$ in comparison to SO, #$p < 0.05$ in comparison to CLP-6h

3.2 Effect on Blood Biochemical Parameters

The effect of sepsis during early and late phases on blood biochemical parameters of mice are presented in Table 2. Sepsis significantly ($p<0.05$) increased the plasma levels of ALT during early and late phases as compared to SO mice. Similarly, there was significant ($p<0.05$) rise in the

AST value in both the early and late phases of sepsis as compared to the SO mice. BUN and creatinine levels were also significantly (p<0.05) increased in both the early and late phases of sepsis as compared to SO mice. But there was significant (p<0.05) decrease in total proteins and albumins level in early and late phases of sepsis as compared to the SO mice. The differences in value of BUN, creatinine, total proteins and albumins between the early and late phases of septic mice were also found to be statistically significant (Table 2).

Table 2. Effect of early (CLP-6h) and late (CLP-20h) phases of sepsis on blood biochemical parameters in mice

Group (n=6)	ALT (U/l)	AST (U/l)	BUN (mg/dl)	Creatinine (mg/dl)	Total Protein (mg/dl)	Albumin (mg/dl)
SO	35.61 ± 5.41	87.51 ± 2.06	25.43 ± 2.27	0.41 ± 0.03	5.46 ± 0.14	3.98 ± 0.12
CLP-6h	72.24 ± 7.52*	175.2 ± 12.52*	96.06 ± 7.92*	0.54 ± 0.02*	4.14 ± 0.24*	2.67 ± 0.12*
CLP-20h	84.30 ± 2.90*	159.6 ± 10.10*	127.5 ± 4.05$^{*\#}$	0.79 ± 0.04$^{*\#}$	2.17 ± 0.31$^{*\#}$	0.99 ± 0.06$^{*\#}$

Data are presented as mean ± SEM, n=6. $^{*}p$<0.05 in comparison to SO, $^{\#}p$<0.05 in comparison to CLP-6h

3.3 Effect on High K^+ (80 mM) Depolarizing Solution-induced Contraction in Mouse Aorta

Endothelium intact aortic rings from SO mice developed sustained contraction (0.52 ± 0.01 g, n=26) to high K^+ (80 mM) depolarizing solution (KDS). This aortic contraction was significantly (p<0.05) reduced in both early (CLP-6h) and late (CLP-20h) phases of sepsis. The KDS-induced aortic contraction in early and late phases of sepsis were 0.23 ± 0.02 g, (n= 26) and 0.27 ± 0.02 g, (n=26), respectively.

3.4 Effect on Vascular Reactivity to Noradrenaline in Mouse Aortic Rings

Figure 1 depicts the vascular smooth muscle reactivity to Noradrenaline (NA) in isolated aortic rings from SO animal as well as early (CLP-6h) and late (CLP-20h) phases of septic mice. The raw tracing (Figure 1A) shows that aortic rings from the SO mice exhibited concentration-dependent contraction to NA (0.1 nM – 1 µM). The pD2 and Emax values of NA in aortic rings of SO mice were 7.82 ± 0.06 and 0.49 ± 0.03 g (n=8), respectively. As illustrated in the Figure 1B and C, the contractile response to NA was significantly (p<0.05) reduced in both early and late phases of sepsis. As compared to the SO mice, the concentration response curves (Figure 1D) of NA were significantly (P<0.05) shifted towards right with decrease in the maximum contractile response (E_{max}) to NA in both early (0.33 ± 0.03 g; n= 7) and late (0.25 ± 0.02 g; n=9) phases of sepsis.

In comparison to the early phase of sepsis, the vascular hyporeactivity to NA was significantly (p<0.05) higher during the late phase of sepsis. However, the potency (pD_2) of NA remained unaltered during both early (8.21 ± 0.14; n=7) and late (7.88 ± 0.13; n=9) phases of sepsis as compared to SO (7.82 ± 0.06; n=8) mice.

3.5 Effect on Endothelium Dependent Aortic Relaxation in Mice

Figure 2 depicts the endothelium-dependent relaxation to acetylcholine (ACh) in phenylephrine (PE) pre-contracted mouse aortic rings from SO animal as well as early (CLP-6h) and late (CLP-20h) phases of septic mice. The raw tracing (Figure 2A) shows that the aortic rings from SO mice exhibited concentration-dependent relaxation to ACh (1 nM – 3 µM). The pD_2 and E_{max} values of ACh in aortic rings of SO mice were 7.41 ± 0.10 and 91.22 ± 1.77 % (n=6), respectively. As illustrated in the Figure 2B and C, the relaxant response to ACh was significantly (p<0.05) reduced in both early and late phases of sepsis. As compared to SO mice, the concentration response curves (Figure 2D) of ACh were significantly (p<0.05) shifted towards right with decrease in the maximum relaxant response (R_{max}) to ACh in both early (63.00 ± 6.49 %; n= 7) and late (43.92 ± 3.01 %; n=7) phases of sepsis.

In comparison to the early phase of sepsis, the relaxant response to ACh was significantly (p<0.05) attenuated during late phase of sepsis. However, the potency (pD_2) of ACh remained unaltered during both early (7.21 ± 0.17; n=7) and late (7.63 ± 0.16; n=7) phases of sepsis as compared to SO (7.41 ± 0.10; n=6) mice.

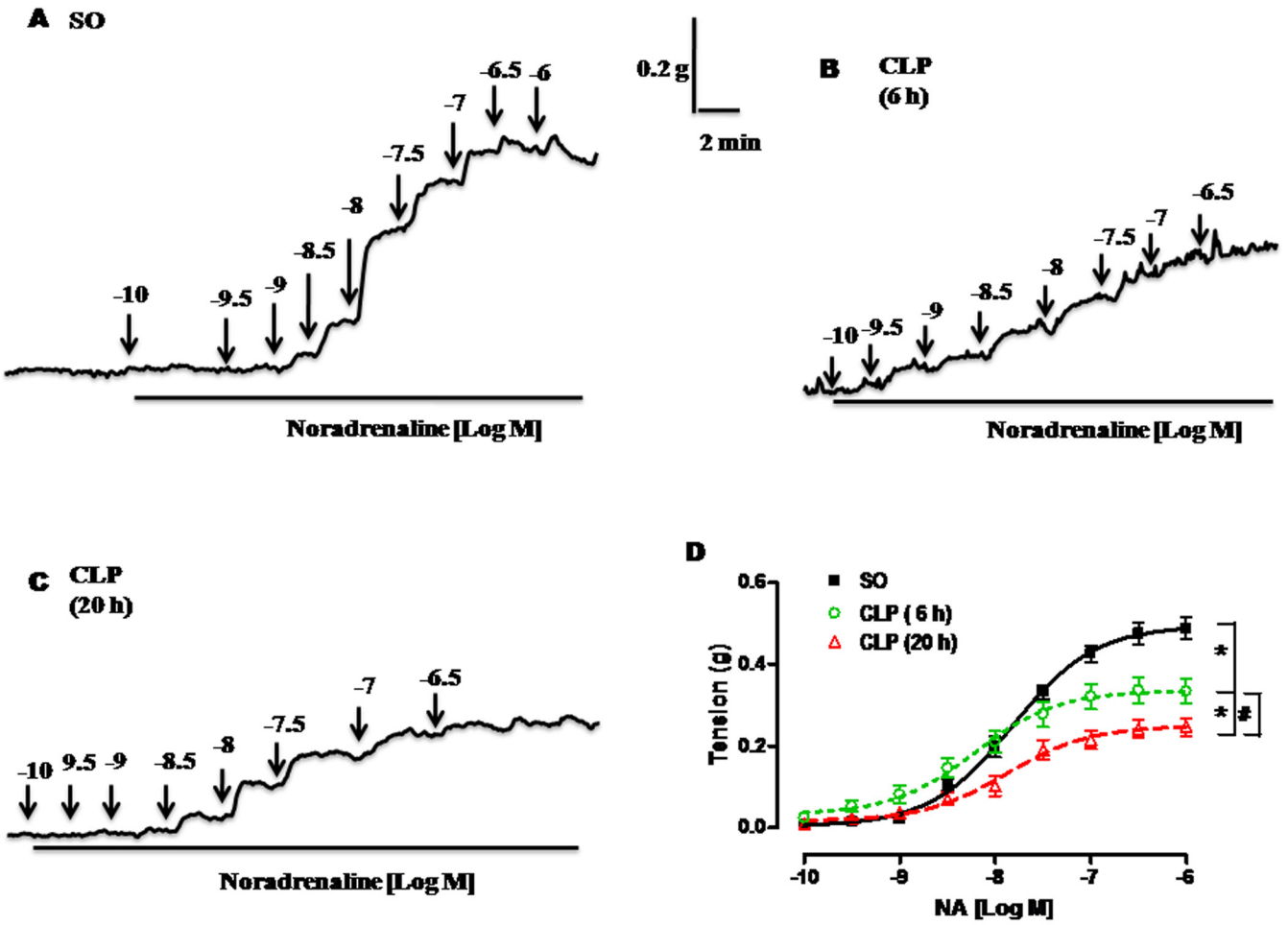


Figure 1. Effect of early (CLP-6h) and late (CLP-20h) phases of sepsis on aortic smooth muscle reactivity to noradrenaline (NA) in mice. Representative tracings show concentration-dependent contractions to NA (0.1 nM – 1 μM) in the aorta taken from sham-operated (SO; A), and early (CLP-6h; B) and late phases (CLP-20h; C) of septic mice. The line diagram (D) depicts the mean concentration-response curves elicited with cumulatively added NA on the aortic rings obtained from different groups. Vertical lines represent SEM. Data were analyzed by two-way ANOVA followed by Bonferroni post-hoc tests. *P<0.05 in comparison to SO; #*p*<0.05 in comparison to CLP-6h.

3.6 Effect on Endothelium-independent Relaxation in Mice

In order to evaluate the involvement of Nitric Oxide (NO) signalling in vascular smooth muscle to impaired endothelium-dependent relaxation in sepsis, we studied the relaxant effect of endothelium-independent nitrovasodilator, Sodium Nitroprusside (SNP), in aortic rings from mice of different groups. Figure 3 shows the concentration-dependent relaxant response to SNP (0.1 nM - 0.3 μM) in Phenylephrine (PE) pre-contracted aortic rings isolated from mice of different groups. The pD_2 and E_{max} values of SNP in SO mice were 8.20 ± 0.18 and 100.00 ± 0.00 % (n=7), respectively. As shown in Figure 3 B, C and D, SNP-induced smooth muscle relaxation was unaffected in both early (CLP-6h) and late (CLP-20h) phases of sepsis as compared to the SO mice. No significant difference was observed either in the pD_2 (7.96 ± 0.11 vs 8.08 ± 0.14) or R_{max} values (100.00 ± 0.00 % vs 98.68 ± 0.97 %) of SNP between early and late phases of sepsis, respectively.

3.7 Effect on Histological Architecture of Vital Organs of Mice

In SO mice, liver section showed normal architecture of the central vein (centre of lobule) and hepatocytes radiating from the central vein towards portal triads (Figure 4A). In contrast, the liver section of early phase (CLP-6h) septic

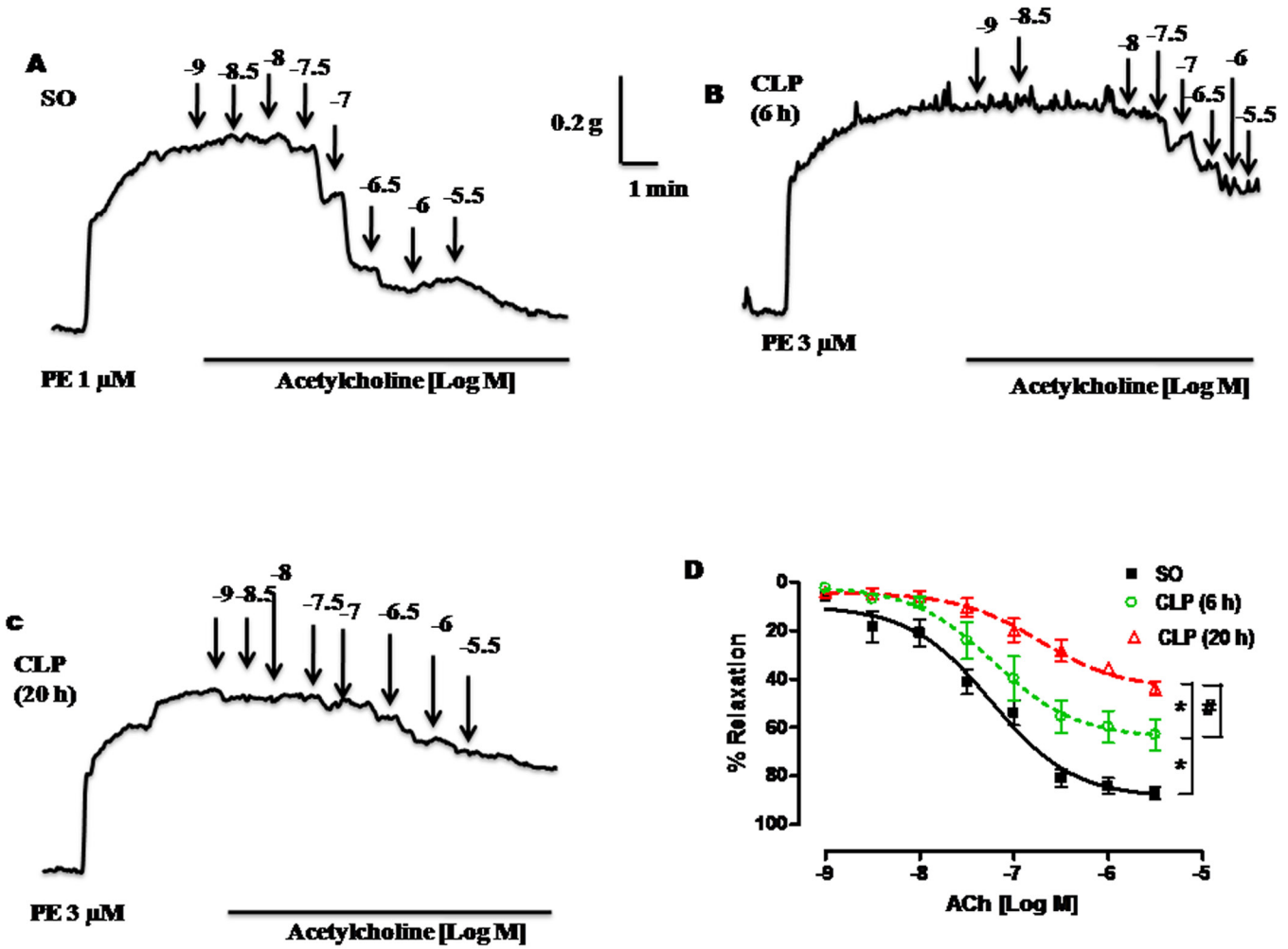


Figure 2. Effect of early (CLP-6h) and late (CLP-20h) phases of sepsis on ACh-induced relaxation in mouse aortic rings pre-contracted with phenylephrine (PE). Raw tracings show the concentration-dependent relaxations to cumulatively added ACh (1 nM – 3 µM) in the aorta taken from sham-operated (SO; A), and early (CLP-6h; B) and late phases (CLP-20h; C) of septic mice. The line diagram (D) depicts the mean concentration-response curves of ACh on the aortic rings obtained from different groups. Vertical lines represent SEM. Data were analyzed by two-way ANOVA followed by Bonferroni post-hoc tests. *p<0.05 in comparison to SO; #p<0.05 in comparison to CLP-6h.

mice showed degeneration of hepatocytes in paracentral area (Figure 4B), while during the late phase (CLP-20h) of sepsis, liver pathological lesions were characterized by severe congestion of central vein along with loss of normal architecture of the hepatic cords and degeneration of hepatocytes (Figure 4C).

Congestion of tubular blood vessels in medulla and glomeruli, infiltration of inflammatory cells and necrosis of tubules and glomeruli was conspicuous in kidney sections of mice during early phase (CLP-6h) of sepsis (Figure 5B). Kidney from late phase (CLP-20h) septic mice also showed remarkable degeneration of tubular and glomerular epithelium along with infiltration of the inflammatory cells and haemorrhages (Figure 5C). In addition, congestion in between the tubules and necrosis of the tubules was also seen.

In early phase of sepsis (CLP-6h), accumulation of edematous fluid in the alveoli with infiltration of inflammatory cells was observed (Figure 6B). In addition, recruitment of inflammatory cells, marked congestion along with thickening of the alveolar septa leading to loss of alveolar structure was also seen. In late phase of sepsis (CLP-20h), pathological lesions include pulmonary edema with infiltration of the inflammatory cells, severe haemorrhages in the bronchiolar lumen along with presence of RBC and exudate were seen (Figures 6C and D). Histopathological examination of heart revealed marked degeneration and necrosis of the muscle fibers

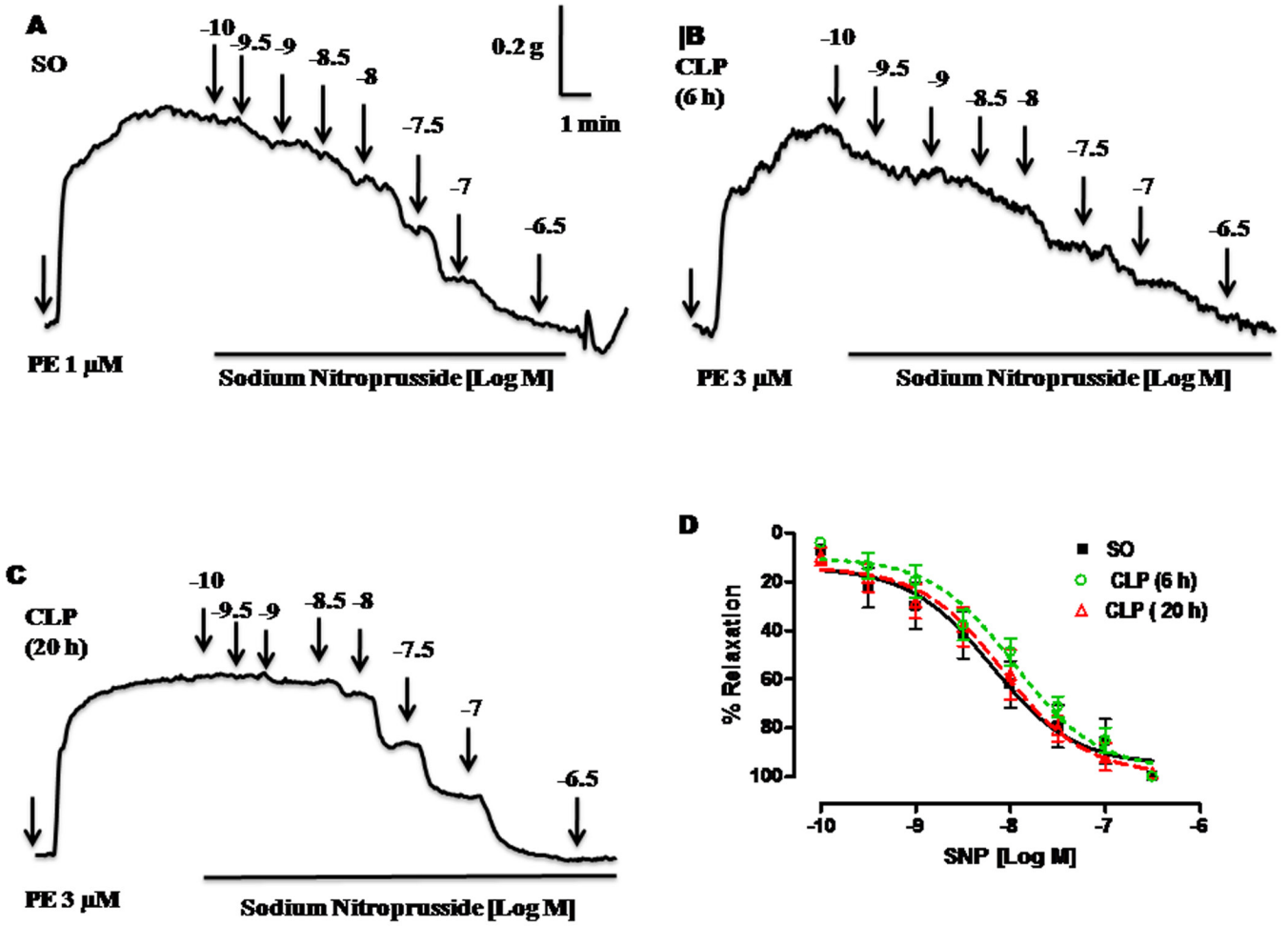


Figure 3. Effect of early (CLP-6h) and late (CLP-20h) phases of sepsis on SNP-induced relaxations in mouse aortic rings pre-contracted with phenylephrine (PE). Raw tracings show the concentration-dependent relaxations to cumulatively added SNP (0.1 nM - 0.3 µM) in the aorta taken from sham-operated (SO; A), and early (CLP-6h; B) and late phases (CLP-20h; C) of septic mice. The line diagram (D) depicts the mean concentration-response curves of SNP on the aortic rings obtained from different groups. Vertical lines represent SEM. Data were analyzed by two-way ANOVA followed by Bonferroni *post-hoc* tests. There was no significant difference between these groups.

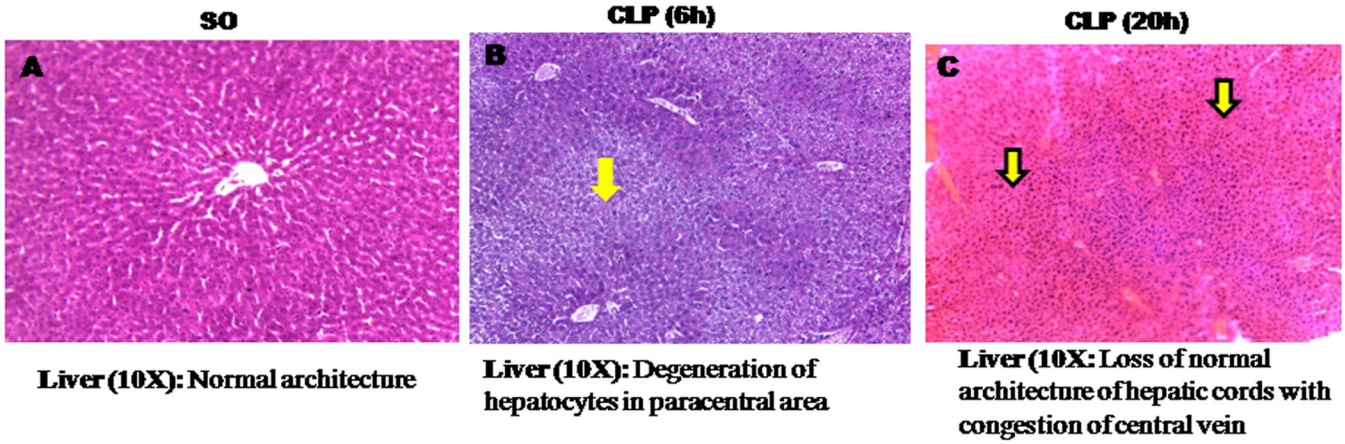


Figure 4. Representative microphotographs of the liver sections of sham-operated (SO; A) mice and early (CLP-6h; B) and late (CLP-20h; C) phases of septic animals.

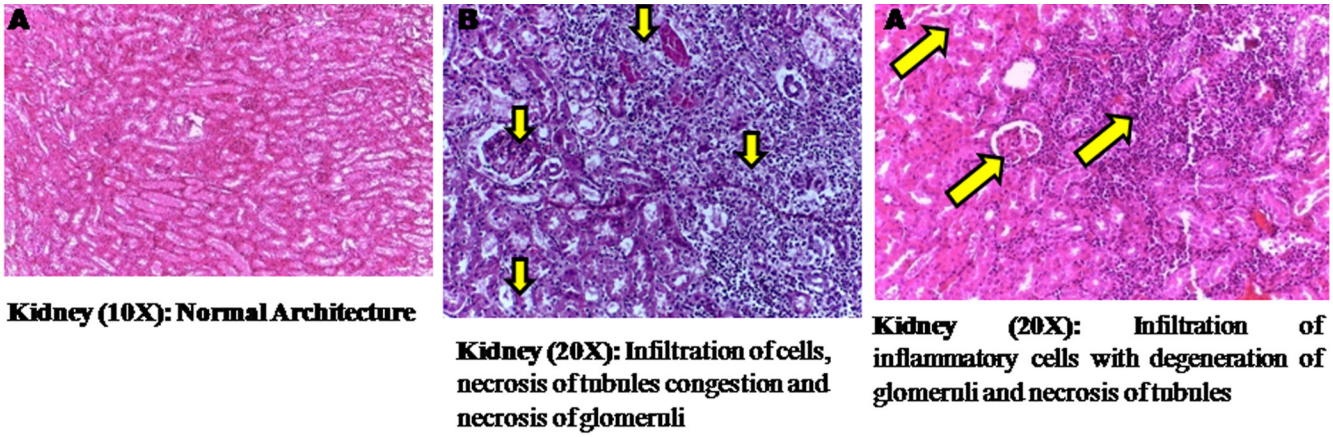


Figure 5. Representative microphotographs of the kidney sections of sham-operated (SO; A) mice and early (CLP-6h; B) and late (CLP-20h; C) phases of septic animals.

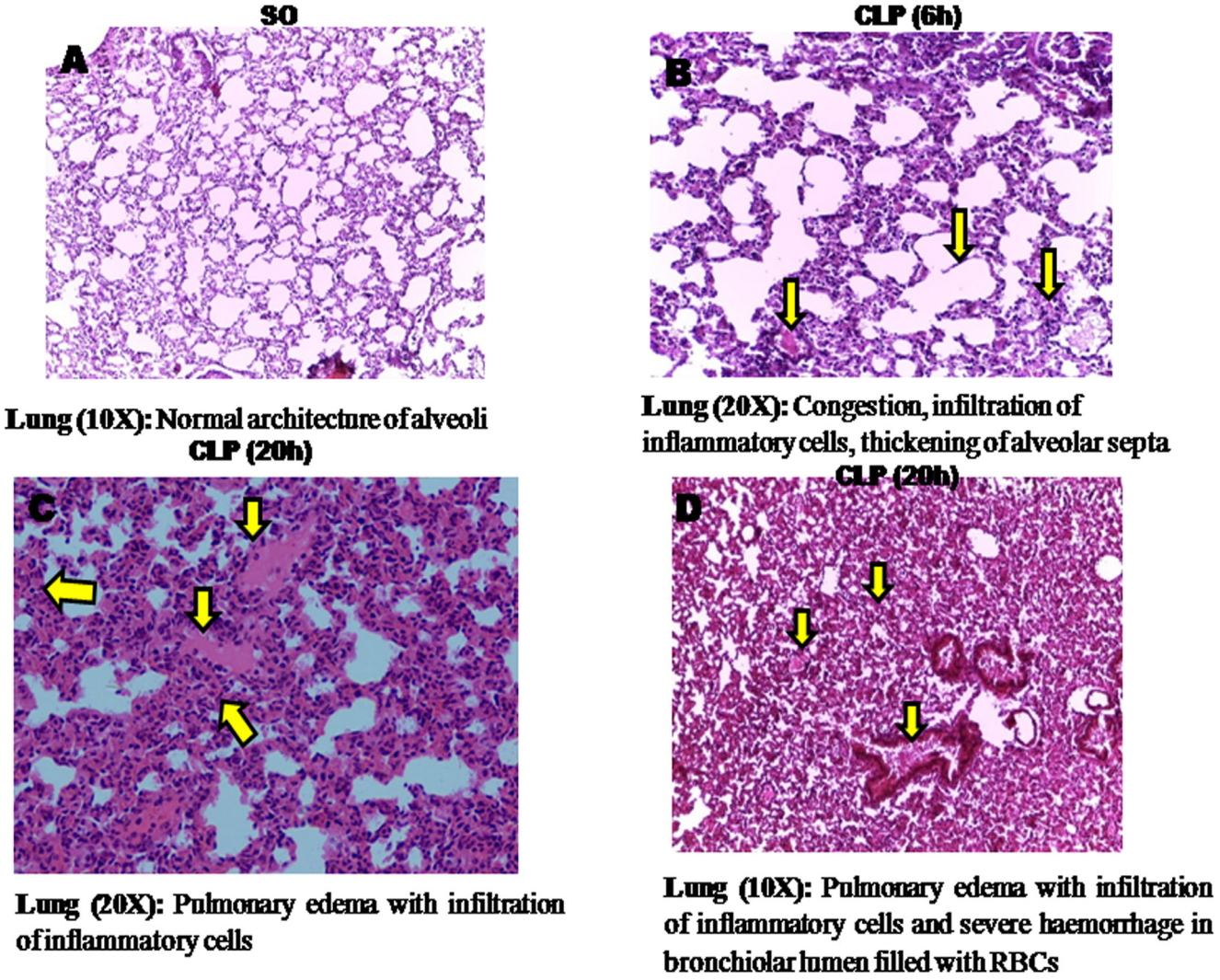


Figure 6. Representative microphotographs of the lung sections of sham-operated (SO; A) mice and early (CLP-6h; B) and late (CLP-20h; C, D) phases of septic animals.

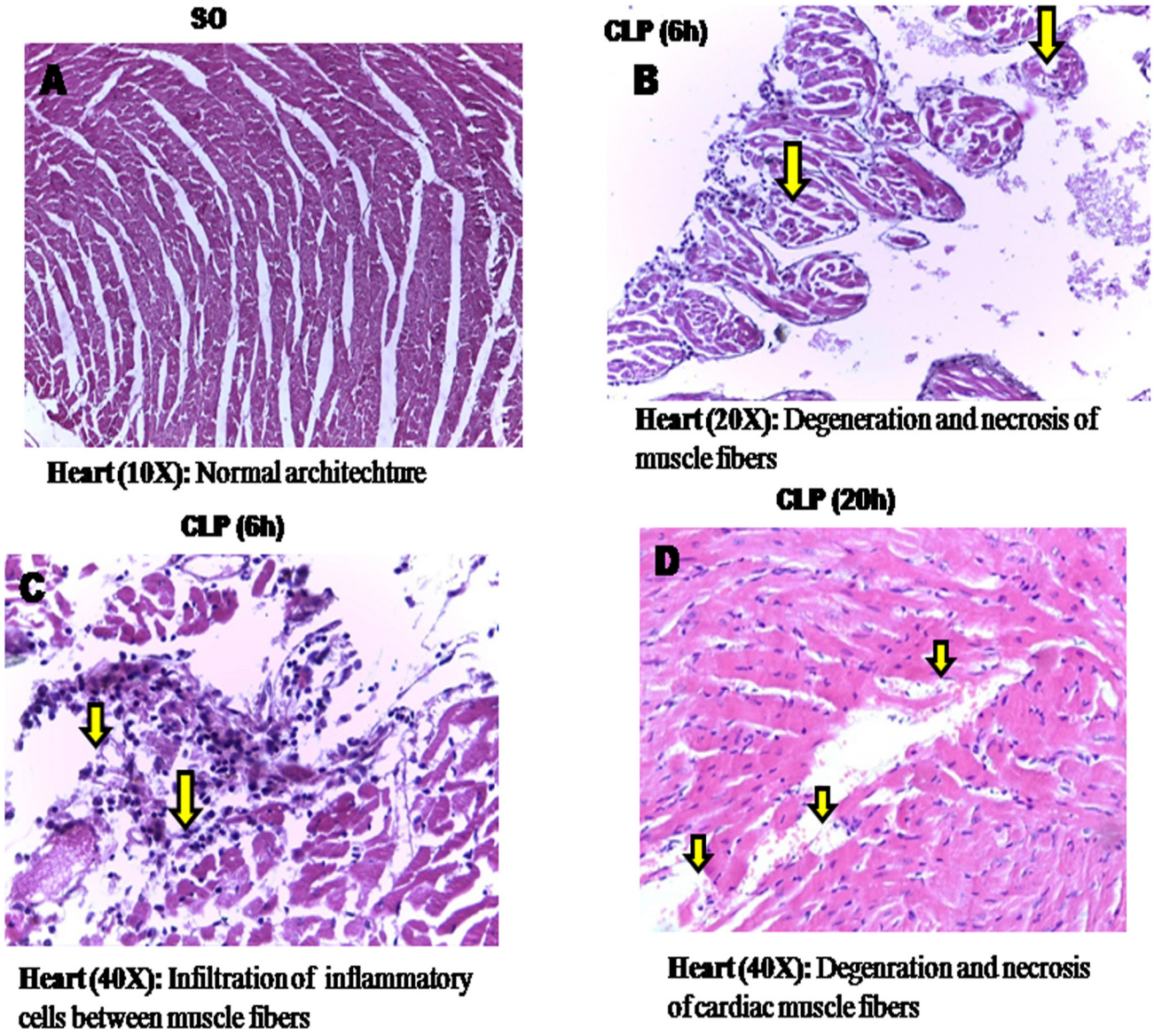


Figure 7. Representative microphotographs of the sections of heart of sham-operated (SO; A) mice and early (CLP-6h; B, C) and late (CLP-20h; D) phases of septic animals.

with infiltration of inflammatory cells between the muscle fibers (Figure 7B, C). During late phase of sepsis (CLP-20h) too, degeneration and necrosis of cardiac muscle fibers were observed (Figure 7D).

In intestinal section of septic mice, detachment of the mucosa, infiltration of the inflammatory cells and degeneration of the crypts and villi of the intestine was observed in early phase (CLP-6h) in septic mice (Figure 8B). In late phase of sepsis (CLP-20h) also, degeneration of the tips of villous epithelium with infiltration of the inflammatory cells in between crypts were observed (Figure 8C). Depletion of lymphoid tissues in the germinal center and congestion of the red pulp were observed in spleen of early (Figure 9B) and late (Figure 9C) phases of septic mice.

Based on the histo-pathological examination, the overall grading of severity of the pathological changes in these vital organs were done as mentioned earlier[7] and are summarized in the Table 3. The scoring was performed by a pathologist who was unknown about the groups.

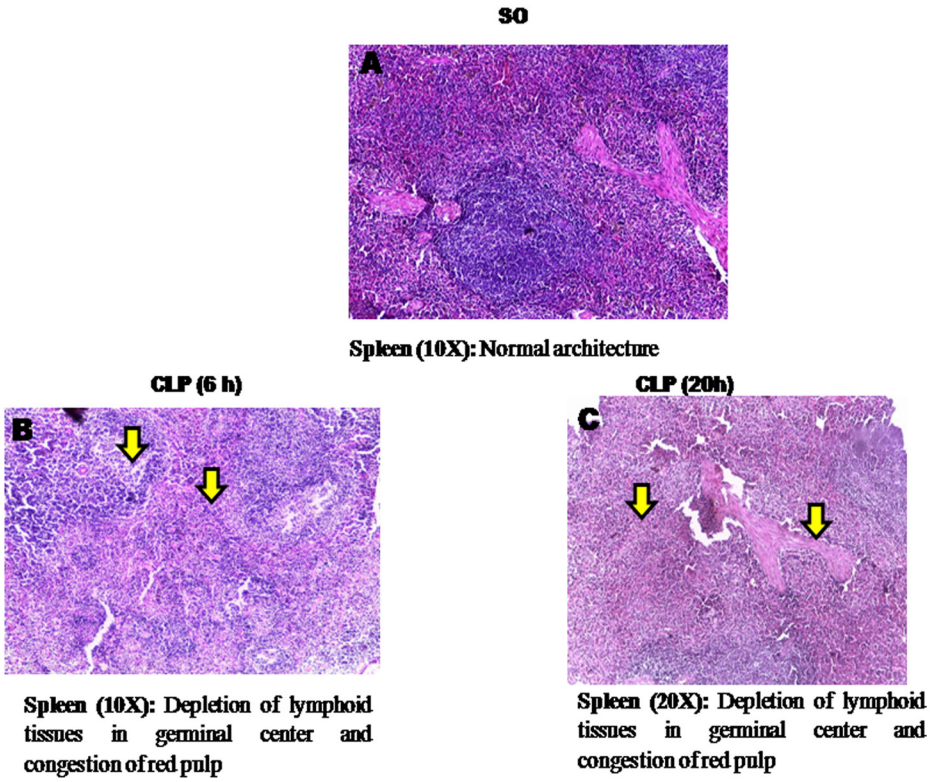


Figure 8. Representative microphotographs of the section of spleen of sham-operated (SO; A) mice and early (CLP-6h; B) and late (CLP-20h; C) phases of septic animals.

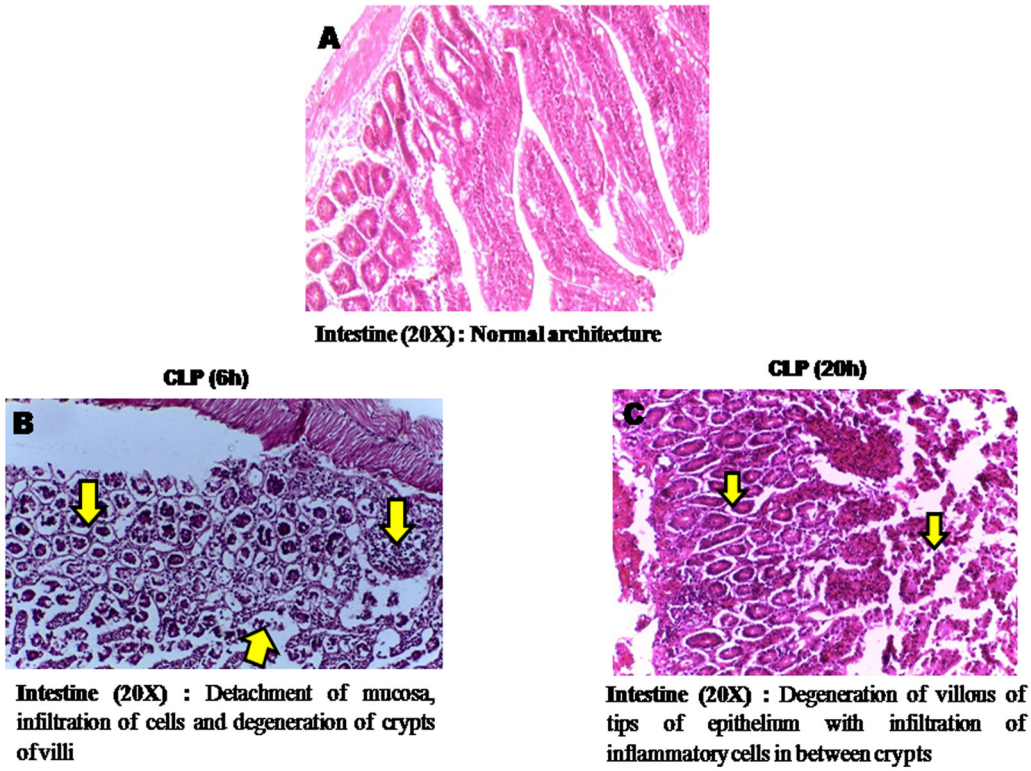


Figure 9. Representative microphotographs of the sections of intestine of sham-operated (SO; A) mice and early (CLP-6h; B) and late (CLP-20h; C) phases of septic animals.

Table 3. Scoring of severity of histopathological changes observed in different organs of animals from different groups

Histo-pathological changes	Liver			Kidney			Lung			Heart			Spleen			Intestine		
	SO	CLP-6h	CLP-20h	SO	CLP-6h	CLP-20h	SO	CLP-6h	CLP-20h	SO	CLP-6h	CLP-20h	SO	CLP-6h	CLP-20h	SO	CLP-6h	CLP-20h
Inflammatory cell infiltration	0	0	0	0	2	2	0	1	2	0	0	2	0	0	0	0	0	1
Degenerations/ Cell injury	0	1	2	0	2	2	0	1	2	0	1	2	0	1	2	0	2	1
Necrosis/cell death	0	2	2	0	2	2	0	2	2	0	1	--	0	1	2	0	1	1
Congestion/	0	1	2	0	2	2	0	--	2	0	0	1	0	1	2	0	0	1
Thrombosis/ Hemorrhage	0	2	3	0	2	2	0	2	2	0	--	0	0	--	--	0	--	--
Epithelial thickening/ Edema	0	0	--	0	--	--	0	1	2	0	--	0	0	--	--	0	--	--

Scoring on the basis of scale, 0=Normal/minimal, 1=Mild change, 2=Moderate, 3=Severe, 4= Very severe; SO= sham-operated, CLP-6h= early phase of sepsis, CLP-20h= late phase of sepsis.

4. Discussion

Sepsis is characterized by multiple organ failure, however, systematic studies on the effect of sepsis on different organs and their functions during early and late phases is lacking. Present study elucidates the time-dependent progression of sepsis following caecal ligation and puncture in mice. This animal model is characterized by increased cardiac output and organ blood flow in early stage (i.e., hyperdynamic phase, 6 h after onset of sepsis[8]) and decreased tissue perfusion at the late stage of hypodynamic phase (18 h after onset of sepsis[9]). In the present study, mean survival time of the septic mice following CLP was 21.40 ± 1.02 h which is in accordance to the earlier findings[10]. Accordingly, we have chosen 6 h and 20 h post-CLP as early and late phases of sepsis, respectively, to study the effect of different phases of sepsis on vital organs of mice.

Septic patients are often diagnosed with leucopenia, increased haemolysis and reduced platelet counts4. In our experimental model of sepsis too, we have also observed significant reduction in RBC, Hb and WBC counts in septic mice. Structural and functional alterations in erythrocytes and reduced levels of haemoglobin impair the microvascular blood flow which results in decrease in tissue oxygen supply in septic patients[11]. Excessive production of reactive oxygen species by inflammatory cells impairs the cellular integrity and contributes to multiple organ dysfunctions. Rise in free hemoglobin level is well reported in animal models of endotoxaemia and sepsis which may be due to increased hemolysis and/or reduced degradation by the endogenous scavenger and detoxification mechanism[12]. Degradation of carbohydrate rich glycocalyx layer is responsible for decrease in haemoglobin concentration[13]. On the other hand, significant ($p< 0.05$) reduction in the total lymphocyte count in septic mice is suggestive of immunosuppression whereas rise in the neutrophil count during early phase is an indication of acute infection.

Refractoriness to vasopressor agents and reduced peripheral vascular resistance are the common features in septic patients[8,9]. Thus to characterize the vascular dysfunction in sepsis, we have first recorded the response of mouse aorta to high K+-depolarizing solution and the vascular reactivity to noradrenaline (NA) in septic mouse. High K^+-depolarizing solution facilitates the entry of extracellular Ca^{2+} into the cell by opening voltage-dependent L-type calcium channels. We have observed that sepsis markedly attenuated contractions induced by high K^+ solution in depolarized aortic preparations during both early and late phases. It is reported that sepsis can activate BK_{Ca} channels in vascular smooth muscle[14] which may inhibit the opening of voltage-gated calcium channel and thereby attenuate vascular

contractility. Further, the vascular reactivity to NA was significantly (p<0.05) and stage-dependently attenuated in our experimental model of sepsis. Again, sepsis-induced vascular dysfunction is associated with endothelial damage resulting in microvascular complicacy. In the present study too, sepsis significantly attenuated ACh-induced relaxation in mouse aorta implicating the endothelial damage which is in agreement with previous findings[15]. Downregulation of eNOS expression and upregulation of iNOS-derived NO production is thought to play a major role in endothelial dysfunction in septic and endotoxaemic model[10]. In contrast to attenuation of ACh-induced relaxation mediated by endothelium-derived NO, sepsis had no significant effect on relaxations elicited by SNP in the mouse aorta, implicating the lack of effect of sepsis on NO/cyclic guanosine monophosphate signaling mechanisms at vascular cell level. Earlier reports also suggest that sepsis does not affect the relaxation at vascular smooth muscle level[10].

Virtually in septic patients every organ may be damaged and multiple organ failure is a recognized complication. The histopathological lesions observed in the lungs, liver, intestine, heart, kidneys and spleen of septic mice are attributed due to abnormalities in microcirculation, capillary leakage following oedema and rapid transfer of bacteria and toxins from the peritoneal cavity into the systemic circulation16. Several inflammatory mediators, pathogens and toxins are released during sepsis may contribute to decrease in tissue perfusion and tissue injury[17]. These histopathological changes observed in the present study during early and late phases of sepsis further substantiate the fact that sepsis reduced the tissue perfusion in patients leading to organ failure.

The reticuloendothelial system of liver plays an important role in clearance of bacteria and bacterial toxins in sepsis and injury to liver aggravates the severity of sepsis by increasing the risk of bacteraemia. Irreversible injury to hepatocytes (hepatocellular dysfunction) integrated with apoptosis and necrosis and accumulation of neutrophils occurs due to hypoperfusion leading to liver damage[18]. Hepatic dysfunction is a substantiated by the level of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), the specific blood biochemical parameters of liver function which were significantly (p< 0.05) elevated in the septic mice during both early and late phases of sepsis as compared to SO mice. These enzymes are released into blood following liver damage and are considered as the biomarker of acute cellular and mitochondrial injury to liver in sepsis[19].

Histopathological lesions observed in kidneys during the early and late phase of sepsis confirm the renal injury during sepsis. Etiologies of renal failure-induced by sepsis is multifactorial, involves decrease in renal perfusion due to endothelial dysfunction, disseminated intravascular coagulopathy and systemic hypotension caused by NO[20]. The exaggerated production of cytokines and free radicals in systemic inflammation might be responsible for renal tubular injury. Renal injury is also substantiated by the significant (p<0.05) progressive increase in BUN and creatinine levels in plasma of septic mice during early and late phases of sepsis[21].

Progressive hepatic and kidney damage observed in the septic mice in the present study are also substantiated by the finding that sepsis significantly (p< 0.05) reduced the plasma levels of total proteins and albumin in affected mice as compared to those in SO mice[22]. Hypoalbuminemia and hypoproteinemia are often associated with sepsis due to reduced synthesis of protein in hepatocytes, increased leakage of protein in interstitial spaces and enhanced catabolism which lead to increased morbidity and mortality[23]. Hepatocytes act as important regulator of albumin synthesis which is adversely affected by acute phase reactions mediated by cytokines in sepsis. However, precise effect of sepsis on plasma albumin in yet to be established[24]. Improper glomerular filtration due to structural damage to glomerulus may also be contributing to severe protein loss and resultant hypoproteinemia.

Acute lung injury is common in septic patients. Loss of endothelial barriers due to release of pro-inflammatory mediators and neutrophil recruitment leading to generation of extravascular traps by formation of toxic metabolites during sepsis[25] causes transition of exudates from vascular lumen to the alveolar spaces resulting in endothelial damage and vascular hyperpermeability, subsequently develop alveolar edema or pulmonary oedema. Extensive damage of epithelium and hemorrhage in alveoli, recruitment of polymorphonuclear cells represent the histological damage in lung injury in CLP-induced sepsis[26]. Now it is widely accepted that the release of inflammatory mediators including free radicals and proteases in acute lung injury play a pivotal role in pathophysiology of sepsis[10].

Hypoxia caused by endothelial damage and thrombosis is responsible for degeneration and necrosis of cardiac muscle fibers[27]. In the present study too, infiltration of inflammatory cells along with degeneration and necrosis of cardiac muscle fibers during early and late stages of sepsis are indicative of the cardiac injury.

Gastrointestinal tract plays a pivotal role in development of multiorgan failure in septic patients. Alteration in internal microenvironment of gut in a pathogenic environment is

associated with sepsis-induced injury[28]. Function of the intestinal epithelium is altered due to gut hypoperfusion and reperfused gut is the source of pro-inflammatory mediators which are responsible for early organ failure. Terminal ileum and caecum are reservoir of gram-negative bacteria and increased production of toxin contributes to sepsis-associated multiorgan dysfunction. Histopathological changes observed in the intestine of septic mice was mainly due to overgrowth of intestinal microbiomes which alter the physiological barrier of intestine due to translocation of bacteria and its toxins into the systemic circulation[29]. Abnormal colonization, gut-associated infections, hyperpermeability of gut, apoptosis of intestinal epithelium, detachment or loss of mucous layer may also contribute in pathogenesis of gut. Some researchers explain gut as a 'motor' of dysfunctioning of several organ[29].

Depletion of lymphoid cells and congestion were observed in spleen of mice during early and late phases of sepsis, however, these changes were less marked compared to the other affected organs. Thus, it may be inferred that in our animal model of sepsis, spleen is less affected compared to other vital organs.

In conclusion, it may be inferred that caecal ligation and puncture produced time dependent progression of sepsis affecting all the vital organs. The changes observed in septic mice during different phases provide a prognostic information for elucidating diagnosis and targeted therapies of sepsis.

5. Conflict of Interest

None of the authors have any conflict of interest.

6. References

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