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Original article

# Immunomodulatory effects of anaesthetic sevoflurane in septic mouse model

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## ABSTRACT

Sepsis is one among the dangerous medical threat that is very much related to body's immune system having no proper treatment for this condition. About 19 million cases of sepsis have been recorded and out of which 5 million cases die every year. Sevoflurane other than controlling the depth of anaesthesia, it does have a vital role in immunomodulations. The study is focused on investigating the immunomodulatory effects of sevoflurane in the septic mouse model induced by CLP. Mortality rate, organ damage, inflammatory mediators, bacterial load, coagulopathy, hepato and renal functional changes, serum lactate, blood glucose, neutrophil sequestration and finally histopathological examination were investigated. The results were interesting that exposure to sevoflurane improves the polymicrobial abdominal sepsis outcome. Mice exposed to sevoflurane after CLP significantly improved outcomes of polymicrobial abdominal sepsis and reduced mortality by improving overall 7-day survival (83.3%) compared to mice without sevoflurane (no treatment group 16.6%) additionally decreasing the surrogate marker levels in the experimental sepsis animal model conducted. Our study suggests that the selection of certain anaesthetic drugs could be critical in the management of septic patients because their immunomodulatory effects could be large enough to affect sepsis pathophysiology.

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## 1. Introduction

Sepsis is one among the dangerous medical threat that is very much related to body's immune system. When a healthy individual get caught by an infection, the immune system in the body develops certain antibodies against the infection to fight it, which fails in case of sepsis and the worst part is that there is not proper treatment for this condition and there by the word life threatening. About 19 million cases of sepsis have been recorded and out of which 5 million cases die every year, a review says (Fleischmann et al., 2016). The measure of the number of deaths due to sepsis is recorded like increasing by 1.5% every year. Resistant bacteria, altered immune system and complicated surgical therapy might

be the possible reasons for the septic cases to rise days ahead (Angus et al., 2001; Hecker et al., 2014; Martin et al., 2003).

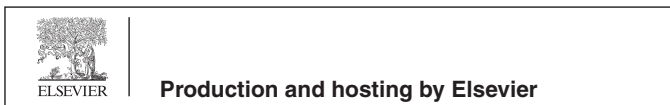
Sevoflurane is the widely used volatile anaesthetic administered through inhalation route. Among the other volatile anaesthetics, sevoflurane's onset of action is much faster and same the offset action. However, general anaesthesia considered to be very much inevitable in septic population on an emergency basis, to remove septic focus, perhaps it decreases the systolic blood pressure and heart rate. Ketamine is one such drug retaining cardiovascular function and in hypoxia where the region is deprived of adequate oxygen supply at the tissue level, it supplies oxygen and thus appears to be the most attractive drug in these circumstances (Baxter, 1997). Sevoflurane is poorly soluble in blood resulting in a faster induction rate and henceforth could be a choice for septic condition regulating the haemodynamics (Manohar and Parks, 1984). It is reported that the blood gas partition rate is 0.69 and so the rapid kinetics of the sevoflurane and the same have been experimented in both preclinical and clinical set up upon many situations and recorded to be much safer drug than the other inhalational agents (Yasuda et al., 1990; Allaouchiche et al., 2001).

Researchers in the field of anaesthesiology proposed general anaesthesia other than controlling the depth of anaesthesia, it do

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have a vital role in immunomodulations (Kurosawa and Kato, 2008). Apoptosis is an important phenomenon in immune system playing a significant role in the control of immune response. In a condition like sepsis, apoptosis occurs primarily in immune cells ending up with drastic immuno modulations. The study is focused on investigating the immunomodulatory effects of sevoflurane in the septic mouse model induced by CLP with this as an underlying reason.

The method developed by Rittirsch et al. (2009) was made used in this study to develop sepsis in animal model and the method is cecal ligation and puncture (CLP). The principle is that releasing the cecal material in to the abdominal cavity will let the bacteria to enter the systemic circulation ending up with systemic inflammatory response syndrome (SIRS) and that will damage the organs one after the other. This is widely used to study the pathophysiology of the sepsis quite similarly happening in humans. With which the inflammatory pathways and the cytokine metabolism is well studied that paves a way to develop the treatment protocol in clinical sepsis (Deng et al., 2017; Doi et al., 2009).

With the aid of CLP induced septic mouse model, mortality rate will be determined to see whether sevoflurane have influence over the mortality to control over. Additionally, body temperature, heart rate and blood pressure were studied. Blood glucose, bacterial load, serum albumin and globulin were measured. The study was conducted to investigate whether sevoflurane volatile anaesthetic has immunomodulatory effects in the CLP mouse model for the treatment of sepsis in the clinical setting.

## 2. Material and methods

### 2.1. Induction of intra-abdominal polymicrobial infection (Sepsis)

The study protocol was approved by the Laboratory Animal Ethical Committee of Weifang Medical University and followed the National Institutes of Health (NIH) Guidelines for the Care and Use of Animals. A method previously described CLP (Rittirsch et al., 2009; Feng et al., 2011), was made used to induce sepsis in mouse model. Animal has to be anaesthetised before the surgical procedure and 60 mg of ketamine and 5 mg of xylazine per kg body weight were made used intraperitoneally. Making sure, the cecum is clearly visible, ligations were performed at a distance of 1.0 cm from its tip and subjected to a single needle using a 18-gauge needle through puncture. The procedure is further followed by removing a trace of fecal matter with a mild pressure, in order to sustain the patency of perforated area. It was then made sure, the cecum relocated back in to the abdominal cavity. To stabilize the tissues, 0.1 mL/g of warm saline was administered subcutaneously. Postoperative surgical pain in the animals undergone surgical procedures were very well expected and to diminish the same, buprenorphine was given subcutaneously.

Animals were segregated in to three groups of six animals each. First group serves as control group and did not undergo any CLP procedure. Second group underwent CLP procedure but not exposed to sevoflurane. Third group underwent CLP procedure and got exposed to sevoflurane. Using sevoflurane vaporizer, group three mice were subjected to expose sevoflurane 1% continuously for 6 h. During anesthetic treatment, mice are constantly examined and fully recovered from anaesthesia.

### 2.2. Survival study

Survival rate was established in CLP groups who were exposed and who were not exposed to sevoflurane. As severe sepsis is a serious condition and the experimental animal mice, tough to

withstand, 7 days survival analysis was studied at an interval of 8 h.

### 2.3. Biochemical analysis

Other than survival analysis, to investigate the immunomodulatory effects of the sevoflurane in mice, blood samples were analyzed to identify the preferable markers responsible for organ damage if any. Inflammatory mediators such as interleukin IL-6, IL-10, tumor necrosis factor TNF- $\alpha$  were measured. 1 mL blood was diluted in 2 mL sterile phosphate-buffered saline. Blood stock solution was made for further analysis which was plated on trypsin blood agar plates (Vijayaraghavan et al., 2019). The diluent was added to the same and the plates were again mounted on an incubator at a temperature of 37 °C and incubated for 24 h. Colonies were counted separately for each sample (Aarti et al., 2017; Arasu et al., 2017). The mean value obtained from the colony count of each sample is multiplied with the dilution ratio and then with a constant 20 to obtain the bacterial load. The major clotting factor platelets (PLTs) were measured under hematology analyzer. Blood samples were collected 0, 8, 16 and 48 h post CLP procedure for the biochemical analysis. Blood biochemical analyzer was introduced to study the biochemical parameters. To study the hepatic functional changes, Alanine aminotransferase (ALT), aspartate transaminase (AST), total protein (TP), globulin and albumin were analysed. To study the renal functional changes, blood urea nitrogen (BUN) and creatinine were analysed. Glucose was also measured. Thromboplastin time, prothrombin time and international normalized ratio, collectively blood coagulation factor was estimated. Serum lactate one other important parameter to assess the sepsis physiological functions, was also estimated. Lactic acid kit was subjected to determine the lactate levels in blood. The principle is that lactate is oxidised to pyruvate and hydrogen peroxide by lactate dehydrogenase eventually interacting with the colorimetric probe to form a red dye whose intensity is measured at 530 nm using a spectrophotometer. Neutrophil sequestration in inflamed tissues is quantified by measuring the tissue myeloperoxidase (MPO) activity (Bradley et al., 1982; Mullane et al., 1985). The liver was isolated 48 h post surgery. The liver tissues was then treated with formaldehyde and stained with hematoxylin and eosin and subjected to histological analysis (Ilavenil et al., 2016; Manonmani et al., 2015).

### 2.4. Statistical analysis

The control group, CLP group and CLP group exposed to sevoflurane were compared among each others for statistical significance. Two way ANOVA with a P value < 0.05 using Graphpad Prism V. 7.0 was used to check the statistical significance. All the data are represented as mean  $\pm$  standard deviation (Al-Dhabi et al., 2020; Vijayaraghavan et al., 2016).

## 3. Results

Exposure to sevoflurane improves the polymicrobial abdominal sepsis outcome. Mice exposed to 1% sevoflurane for 6 h after CLP significantly improved outcomes of polymicrobial abdominal sepsis and reduced mortality by improving overall 7-day survival (83.3%) compared to mice without sevoflurane (no treatment group 16.6%) when treated with saline at a dosage of 0.015 mg / g immediately following CLP surgery (Fig. 1). At day 3, 100% of mortality was observed with the septic mouse not exposed to sevoflurane. As the condition sepsis is a bacterial infection, bacteria from these infections enter the systemic circulation at once and start proliferating. Symptoms can be notified much quicker when

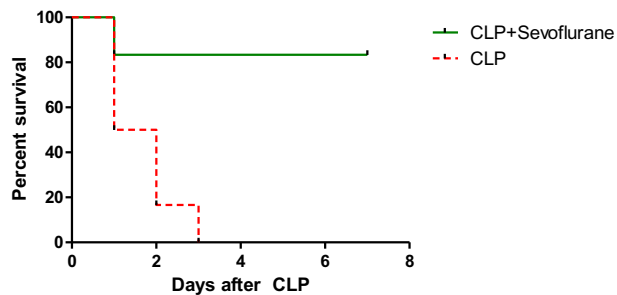


Fig. 1. Kaplan-meier curve showing the percentage survival. Survival analysis observed for seven days among the mice undergone CLP and CLP mouse exposed to sevoflurane (N = 6).

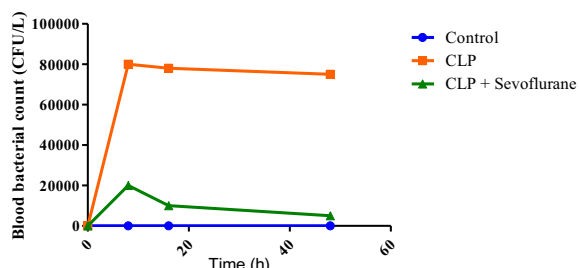


Fig. 2. Bacteria load observed among the control group, mice undergone CLP and CLP mouse exposed to sevoflurane (N = 6).

the bacteria got circulated and multiplied in the blood stream. Hence, it is important in septic infection to watch closely the blood bacterial load as it has a close relationship with high mortality. At increased bacterial load, mortality rate increased. The bacterial load in blood post-CLP 0, 8, 16, and 48 h was examined. The bacterial load, as demonstrated in Fig. 2, a significant increase is noted after 8 h and stayed high for 48 h. The bacterial infection causes Immune system dysfunction in patients with sepsis. As expected, a decrease in the number of leukocytes and lymphocytes was

observed in the CLP group not exposed to sevoflurane throughout the study period. The same was not observed in the CLP group exposed to sevoflurane however lesser count noticed at the early stage. Higher bacterial load was observed in CLP group not exposed to sevoflurane than the group subjected to sevoflurane. In the CLP group not exposed to sevoflurane, PT and APTT were significantly high 48 h post CLP. PLT level was decreased significantly throughout the observation period as shown in Fig. 3. The APTT, PT and PT-INR significantly increased from 16 h post CLP surgery throughout the study period in the CLP group and the same is presented in Fig. 3. In the cases of control and CLP group exposed to sevoflurane, there was no significant difference in the levels of coagulation factors. At 8 h post-CLP, levels of IL-6, and TNF- $\alpha$  (Fig. 4) increased significantly with a decline in the later hours. No significant differences were found in inflammatory mediators among the control and sevoflurane exposed group. As far as hepatic function is concerned, both ALT and AST (Fig. 5) increased gradually from 0 to 16 h post-CLP in animals which were not exposed to sevoflurane and these levels found retained through the experimentation. The levels of total protein and globulin decreased from 0 to 48 h as shown in Fig. 5. Furthermore, over the 48 h observation period, the albumin level remained significantly low as shown in Fig. 5. The differences in the levels of creatinine and blood urea nitrogen are fixed on with the glomerular filtration rate with respect to renal function. Renal dysfunction led to higher rates of creatinine and blood urea nitrogen. The levels of blood urea nitrogen and creatinine (Fig. 6) increased gradually from 0 to 16 h post-CLP in animals who were not exposed to sevoflurane. The levels of serum glucose (Fig. 7) decreased significantly 8 h post-CLP and the levels were maintained throughout the experimentation. But in case of sepsis where, decreased tissue oxygenation exists, pyruvate will not be in a position to metabolize much faster and there by the intracellular levels rise eventually leading to increased levels of lactate. Due to the metabolic changes caused by hypoxia, the serum lactate level increased and the same is represented in Fig. 8. Interestingly, the levels decreased at 8 h, and there after no changes noted at 16 and 48 h. In the CLP group, liver histopathology shows a loss in liver tissue organization and structure, necrosis and inflammatory cell infiltration, and hepatocyte

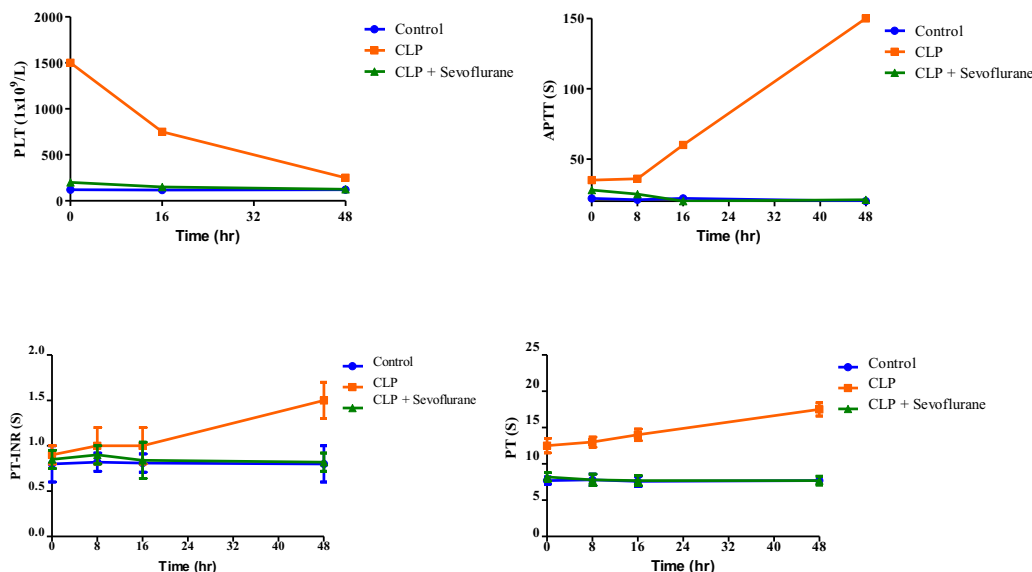


Fig. 3. Coagulation parameters. Platelet (PLT), Activated partial thromboplastin time (APTT), Prothrombin time – international normalised ratio (PT-INR), Prothrombin time (PT) observed among the control group, mice undergone CLP and CLP mouse exposed to sevoflurane (N = 6).

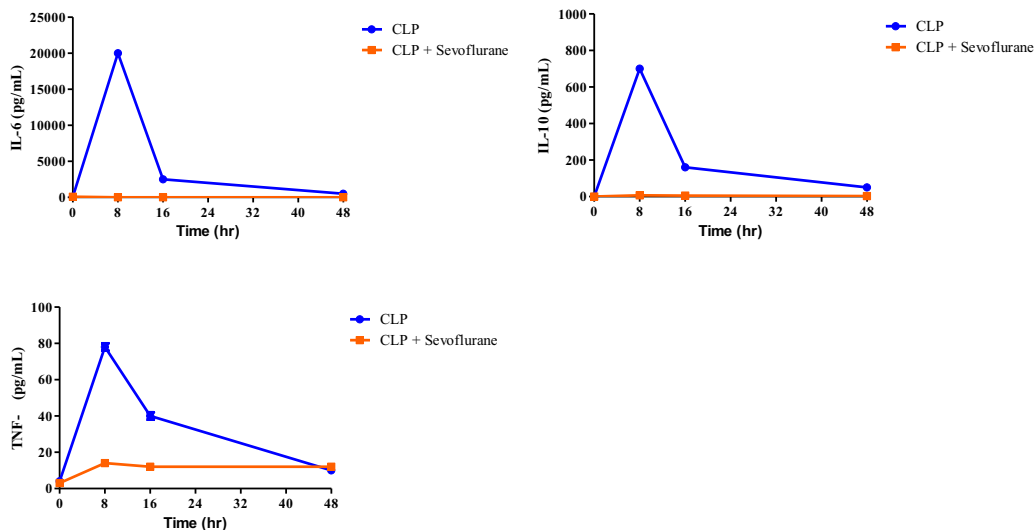


Fig. 4. The inflammatory parameters. IL-6, IL-10, TNF- $\alpha$  observed among the control group, mice undergone CLP and CLP mouse exposed to sevoflurane (N = 6) (IL, interleukin; TNF, tumor necrosis factor).

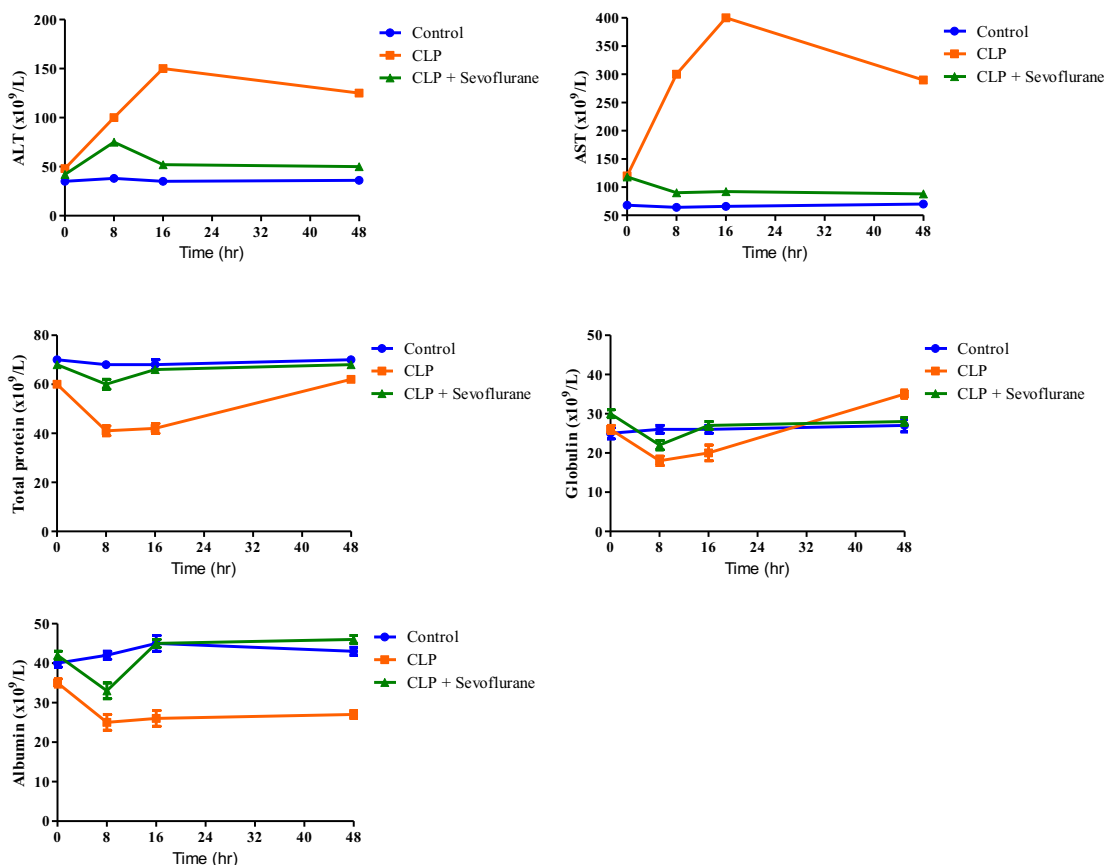


Fig. 5. Hepatic function 5(a) ALT, Alanine Transaminase; 5(b) AST, Aspartate transaminase; 5(c) Total protein; 5(d) Globulin; 5(e) Albumin observed among the control group, mice undergone CLP and CLP mouse exposed to sevoflurane (N = 6).

degeneration. There was an abnormal narrowing of the central vein. Nevertheless, the CLP group subjected to sevoflurane had no or slight histological changes (Fig. 9). In the present study, neutrophil was quantified in the inflamed spleen and lung tissues by measuring the tissue MPO activity. MPO activity was increased

3.5 fold in the spleen tissues of CLP group compared to CLP group exposed to sevoflurane and the control group. Also, 2.2 fold increase of MPO activity was observed in the spleen tissues of CLP group compared to CLP group exposed to sevoflurane and the control group (Fig. 10).



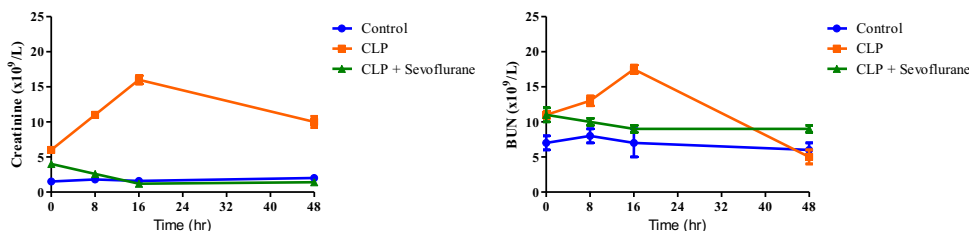


Fig. 6. Renal function 6(a) BUN, Blood urea nitrogen; 6(b) Creatinine observed among the control group, mice undergone CLP and CLP mouse exposed to sevoflurane (N = 6).

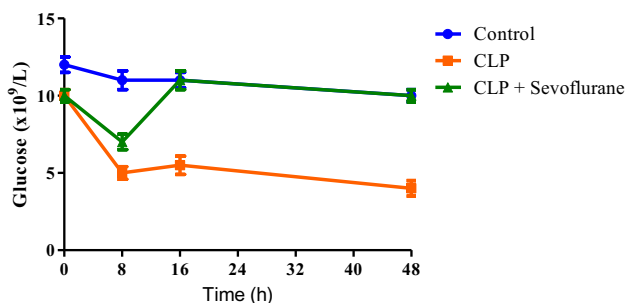


Fig. 7. Blood glucose level observed among the control group, mice undergone CLP and CLP mouse exposed to sevoflurane (N = 6).

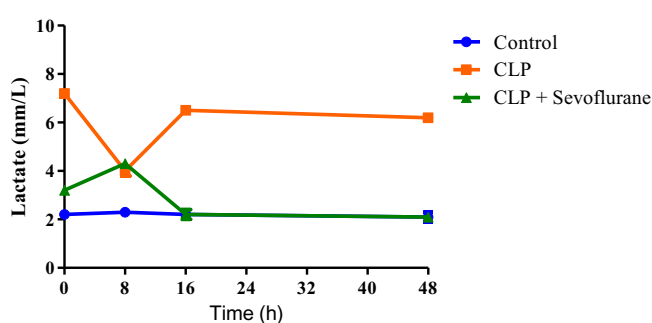


Fig. 8. Serum lactate observed among the control group, mice undergone CLP and CLP mouse exposed to sevoflurane.(N = 6).

4. Discussion

In sepsis, one other important characteristic phenomenon is coagulation. Coagulation here in sepsis, is immune system response to infection and that would get advanced to intravascular coagulation. It is a condition where, the many a small blood clots would develop throughout the systemic circulation eventually blocking the blood vessels. It is one other cause for the multiple organ damage. Henceforth as discussed earlier, this would again increase the mortality rate in sepsis cases. Normally PT and APTT were studied as coagulation factors. DIC often causes sepsis (Rittirsch et al., 2009; Levi and van der Poll, 2010; Li, 2015). As like bacterial load, excessive the stimulation of coagulation increased the mortality in sepsis cases. The data is so convincing that the bacteria entering the systemic circulation, multiplies and provoke the inflammatory response and the inflammatory markers here play a major role in the pathogenesis of sepsis. Cellular mediators like Lipopolysaccharide (LPS), Lipoteichoic acid, Peptidoglycan, super antigens, endotoxin and humoral mediators activates macrophages, neutrophils, platelets, and releases various cytokines and

other mediators. They do activate inflammatory pathways. These mediators act as a foreign body to the biological system activating the proliferation of B cells and T cells. In the present study, IL-6, IL-10 and TNF- α were evaluated. As discussed earlier, activation of cellular and humoral inflammatory mediators influences a lot in the pathological changes of hepatic and renal physiology. To understand the physiological changes of hepatic and renal functioning, serum biochemical analysis was performed in CLP mouse model. No significant differences were found with respect to hepatic and renal functions among the control and sevoflurane exposed group.

Serum lactate is a glycolysis metabolite. In sepsis, the rate of blood lactate represents the cell hypoxia stage and is an important marker predicting mortality (Wang, 2014). Lactate is a substance that is usually produced by body cells when the food gets converted to energy and these do exist in the cells. Lactates existing in the cells move around and reaches liver whenever needed. Lactates reaching to the liver will get oxidized to pyruvate and even-

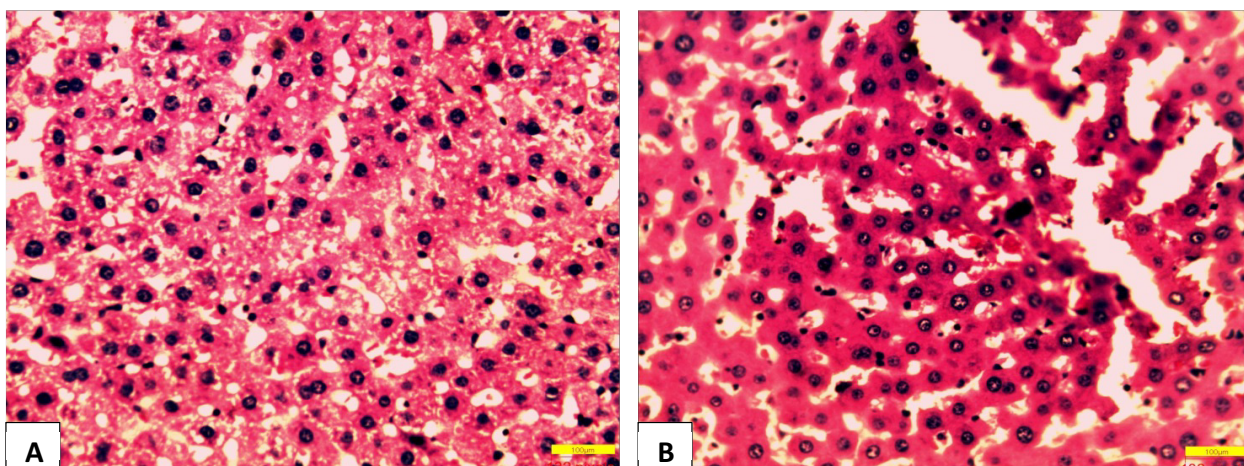
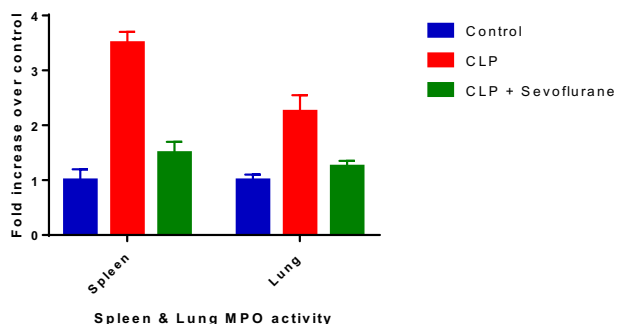


Fig. 9. Histological examination (a) Liver tissues of CLP mice exposed to sevoflurane (no necrosis, inflammatory cells and degeneration of hepatocytes); (b) Liver tissues of CLP mice. Necrosis and infiltrations of inflammatory cells were observed. Degeneration of hepatocytes was observed, Irregular central vein congestion were observed.



**Fig. 10.** Quantification of neutrophil in inflamed spleen and lung tissues by measuring the tissue MPO activity.

tually to glucose through cori cycle. As lactate can be much rapidly converted to pyruvate and get in to the recyclization, all the tissues in need can utilize lactate as an energy source. As simple as to say, lactate get oxidised to pyruvate and pyruvate get metabolize rapidly. Both the control and the sevoflurane exposed group remains the same with no significant differences in the serum lactate levels. By the cell-death process known as apoptosis, they also had slightly fewer depletion of neutrophils in the spleen and lungs. The assessment of tissue MPO activity quantifies neutrophil sequestration in inflamed tissues. MPO is a hemoprotein stored in polymorphonuclear neutrophils and macrophages in azurophilic granules. MPO catalyzes chloride and hydrogen peroxide conversion to hypochlorite and is secreted during inflammatory condition by activating neutrophils. In summary, inhalation sevoflurane administration not only decreases surrogate marker levels, but also increases outcome in the experimental sepsis animal model conducted. Our study suggests that the selection of certain anesthetic drugs could be critical in the management of septic patients because their immunomodulatory effects could be large enough to affect sepsis pathophysiology.

## 5. Conclusions

To conclude, the data obtained shows beneficial effects of inhalation anaesthetic sevoflurane in a sepsis model on survival with improved results. This is the first experimental study to collectively demonstrate a survival benefit with all the immunomodulatory effects including inflammatory mediators, coagulation factor, hepatic and renal functions, neutrophil estimation and histopathological changes by administering a volatile anesthetic sevoflurane in the septic mouse model induced by CLP. Clinical data, however, are not available to support more. The findings should be incorporated in clinical setting and the authors will come up with the clinical data in near future. Clinicians should take up sevoflurane trying the same as a therapeutic choice for the treatment of sepsis. In surgery for people with sepsis, choice of anaesthetic matters a lot and selection of sevoflurane volatile anaesthetic could be critical in the management of sepsis.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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