EXPERIMENTAL STUDY

Effect of fluid resuscitation on acute lung injury in a rat model of sepsis

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ABSTRACT

AIM: Sepsis is a systemic infection reaction and intravascular volume therapy plays a crucial role in it's treatment. Acute respiratory distress syndrome (ARDS) occurs in the lungs, the most affected organ. This study aimed to investigate the different effects of fluid therapy on ARDS caused by sepsis.
METHOD: To form a sepsis model, cecal ligation and puncture (CLP) procedure were performed on 44 adult rats. Divided into six groups; normal, CLP group, those treated with 40 ml/kg 0.9 % NaCl, 3 % NaCl (hypertonic saline), Ringer Lactate and Hydroxyethyl starch. After 24 hours treatments, histopathological examination of the lungs were done, and the plasma levels of CRP, TNF-α and IL-6 and paO_2 were measured.
RESULTS: The scores of all histological parameters of the group treated with hypertonic saline were significantly lower than of the other groups (p < 0.001). Likewise, according to the arterial blood gas results, paO_2 was significantly higher (p < 0.01) in the hypertonic saline group compared to the other groups, and $paCO_2$ was significantly lower (p < 0.01). CRP, TNF-α and IL-6 levels of inflammatory markers were also significantly lower in hypertonic saline groups compared to other groups (p < 0.001).
CONCLUSIONS: Our study shows that treatment with hypertonic saline reduces the progression of ARDS in sepsis *(Tab. 3, Fig. 4, Ref. 49).* Text in PDF *www.elis.sk*
KEY WORDS: ARDS, inflammation, lung injury, sepsis, volume replacement fluids.

Introduction

Sepsis, which is an erratic response of the host to infection, is a life threatening disease with a globally high mortality rate (1). Severe sepsis is a suspected or documented presence of infection with signs of systemic response which is accompanied by organ dysfunction (2). Mortality rates from serious sepsis exceed 25 % to 45 % in the case of septic shock, even with optimal treatment (2). Therefore, in order to perform the clinical management of sepsis in the best way, the resuscitation method should be selected as the most appropriate procedure.

Systemic inflammatory response syndrome, which usually begins with severe infection or acute injury, is a common problem in sepsis (3, 4). This syndrome has also caused multiple organ dysfunction, including the lungs, liver, heart and kidneys (3, 5). The lungs are the most commonly affected organ in multiple organ dysfunction syndrome due to sepsis, and this syndrome takes the form of acute respiratory distress syndrome (ARDS) with lung injury (6, 7).

Although there are significant progresses in our experience about the pathophysiology of sepsis, treatments are still limited to aggressive fluid resuscitation, antibiotics, vasopressor administration and supportive care (8). Therefore, it is highlighted that there is a need to develop new therapeutic strategies for this syndrome. Intravenous (IV) fluid resuscitation is a critical component of the ideal treatment plan for serious sepsis patients (2). Consequently, the effect of various types of IV fluids has also been a popular subject of interest in sepsis studies and research (2). For all that it is still controversial about the use of optimum fluid replacement especially with regard to the kidney function. For example, though in European emergency clinics, gelatin and hydroxyethyl starch are generally used combination with crystalloids for fluid resuscitation, in North America crystalloids can be used frequently (9–12).

In sepsis induced ARDS and/or Acute Lung Injury (ALI), the lungs are the most severely inflamed and damaged areas (13, 14). The main cause of damage in ARDS is the destroying of the alveolar epithelium and pulmonary capillary endothelium by activated neutrophils, leukocytes, and macrophages, which form a large

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number of inflammatory mediators, interleukins (ILs), including tumor necrosis factor (TNF)-α (15). Thus, inflammatory processes must be evaluated regarding this information. Fluid resuscitation plays an essential role in septic tissue hypoperfusion therapy of hypovolemia (16). For this reason, this study was planned to research the effects of volume replacement by IV fluids on sepsis induced ARDS model in rats.

Materials and methods

Animals

In our study, 44 Sprague Dawley albino mature 200–220 g male rats were used. We received local Animal Ethics Committee's approval. And then all experimental procedures were performed according to the stated rules in the Guide for the Care and Use of Laboratory Animals adopted by the National Institutes of Health and ARRIVE guidelines. Animals were fed and kept in temperature – controlled steel cages (21 ± 2 °C) light-dark period for 12 hours.

Experimental procedures

In order to create a sepsis model, cecal ligation and puncture (CLP) procedure were performed on 44 rats as previously described (16, 17). 8 rats were excluded from the study because they died. We randomly divided the rats into six groups. We designed the working groups as follows: Group 1: normal (non-operative and orally fed control, n = 6); Group 2: CLP (untreated group, n = 6); Group 3: CLP and 40 ml/kg 0.9 % NaCl i.p (n = 6), Group 4: CLP and 40 ml/kg 3 % NaCl i.p. (n = 6), Group 5: CLP and 40 ml/kg Ringer Lactate i.p (n = 6), Group 6: CLP and 40 ml/kg Hydroxyethyl starch (6 % HES 130/0.4) i.p. We anesthetized the rats by intraperitoneally administering a combination of ketamine hydrochloride (80 mg/kg) and xylazine hydrochloride (7 mg/kg) according to the CLP procedure. Aseptically, the cecum was reached by applying 3 cm laparotomy from the midline of the rat. It was tightly tied at the base of the cecum with a 3.0 silk suture and pierced with a 22-gauge needle. A small amount of stool was then squeezed out of the puncture site. The cecum was placed back into the peritoneal cavity and the laparotomy incision was closed with 4-0 polyglactin sutures. Treatment was started in the first hour of the surgical procedure. All volume replacement therapies were given equally every 12 hours a day in two sections. All treatments were administered for 24 hours. When we ended the study, the rats were euthanized, biochemical analysis was performed from the blood sample taken by cardiac puncture, and histopathological examination was performed by pneumonectomy.

Measurement of Arterial blood gas

Carotid artery blood (0.2 mL) of rats was taken and paO_2 and $paCO_2$ levels were evaluated.

Measurement of plasma CRP, TNF-α and IL-6 levels

Biochemical analysis was performed from blood samples collected by cardiac puncture after the sacrifice procedure. Blood samples were centrifuged at 3000 rpm for 10 minutes at room temperature and then stored at –20 °C. Commercially available enzyme-linked immunosorbent assay (ELISA) kits were used to evaluate CRP, TNF-a, and IL-6 levels. We identified all samples in duplicate according to the manufacturer's instructions.

Histopathological examination of lung

All rats were anesthetized by ketamine (40 mg/kg, Alfamine, Alfasan International B.V., The Netherlands) and xylazine (4 mg/kg, Alfazyne, Alfasan International B.V., The
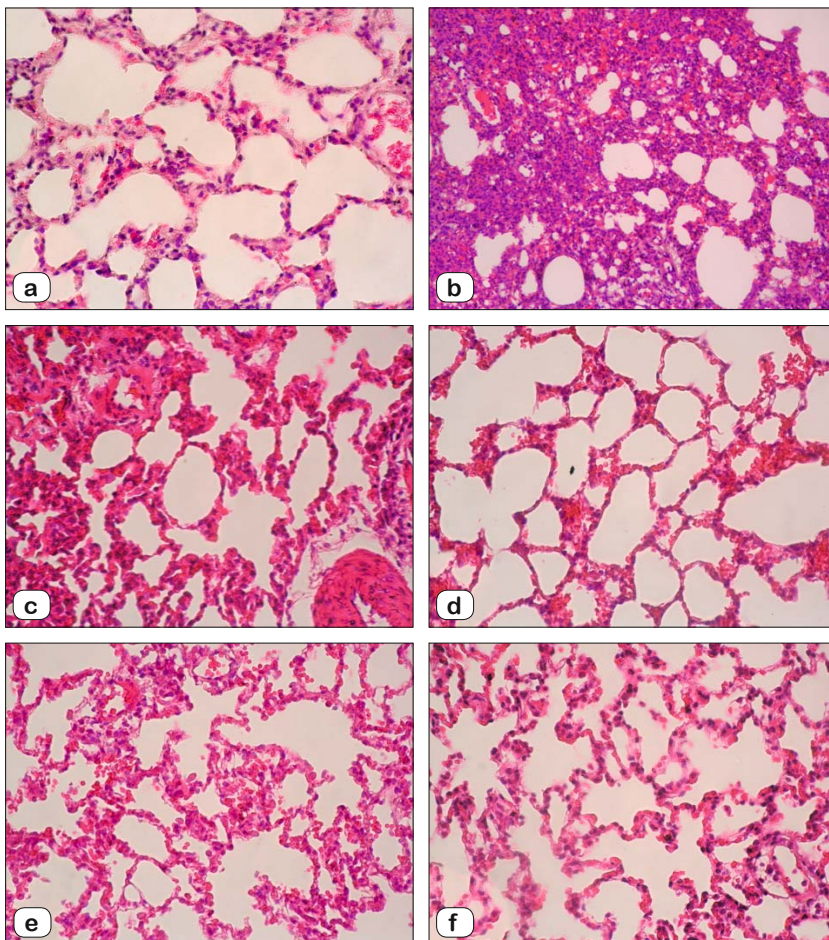
Fig. 1. Lung Tissue. Hematoxylin and Eosin (H&E) stain, X 20 magnification. a: Control group, normal alveolar and interstitial structure, b: CLP group, c: CLP and 0.9 % NaCl group, d: CLP and 3 % NaCl group, e: CLP and Ringer Lactate group, f: CLP and HES group.

Tab. 1. Changes in histopathological lung injury scores.

	AC	H	AL	PE	TA
Control group	0.3±0.2	0.5±0.2	0.6± 0.3	0.5±0.2	0.3±0.2
CLP group	4.3±0.4*	3.3±0.3*	4.3±0.4*	3.8±0.4*	4.3±0.4*
CLP and 0.9% NaCl group	3.5±0.2#	2.0±0.3#	3.3±0.3#	2.3±0.2#	3.1±0.4#
CLP and 3% NaCl group	1.6±0.1##	1.7±0.4##	1.3±0.2##	1.5±0.2##	1.6±0.4##
CLP and Ringer Lactate group	2.6±0.3#	2.2±0.3#	1.5±0.2##	1.7±0.2##	2.2±0.3#
CLP and HES group	2.0±0.3##	1.8±0.2##	3.0±0.2#	1.8±0.3##	2.0±0.4##

* $p < 0.0001$, compared with Control group, # $p < 0.05$, compared with CLP group, ## $p < 0.001$, compared with CLP group. Alveolar congestion (AC), hemorrhage (H), infiltration or aggregation of leukocytes in air spaces/vessel walls (AL), perivascular/interstitial edema (PE), and thickness of the alveolar wall/hyaline membrane formation (TA)

Tab. 2. Arterial blood gas analysis.

	paO$_2$ (mmHg)	paCO$_2$ (mmHg)
Control group	121.±5 1.7	36.6±1.3
CLP group	72.3±5.6*	55.3±4.2*
CLP and 0.9% NaCl group	94.8±7.4#	44.5±4.7#
CLP and 3% NaCl group	110.±6 4.7##	39.5±3.3##
CLP and Ringer Lactate group	95.6±5.4#	45.1±1.3#
CLP and HES group,	99.3±7.3##	43.5±1.8#

* $p < 0.001$, compared with Control group, ## $p < 0.01$, compared with CLP group, # $p < 0.05$, compared with CLP group

Netherlands) i.p. Lungs were removed for histological examination and fixed with formalin. During the examination, it was stained with hematoxylin/eosin. All stages were taken by the Olympus C-5050 digital camera mounted on the Olympus BX51 microscope. The basic histopathological lung injury score was calculated as used in previous publications (18). In a nutshell, histopathological lung damage was assessed by scoring alveolar congestion (AC), hemorrhage (H), infiltration or aggregation of leukocytes in air spaces/vessel walls (AL), perivascular/interstitial edema (PE), and thickness of the alveolar wall/hyaline membrane formation (TA) (18). For all cases, the significance was graded on a scale of 1 (0–25 %), 2 (25–50 %), 3 (50–75 %), and 4 (75–100 %).

Statistical analysis

The data obtained from the subjects were compared with one-way analysis of variance (ANOVA). Post-hoc Tukey HSD test was used for comparison of the groups. Differences in control and treatment values were determined using Student's t-test. The limit of statistical significance was assigned to $p < 0.05$.

Results

Hitopathological examination of lung

Lung tissue histopathology changes are shown in Figure 1. Histopathological indicators of ARDS (hemorrhage, alveolar conges-

tion, infiltration/ aggregation of leukocytes in air spaces-vessel walls, perivascular-interstitial edema, and thickness of the alveolar wall-hyaline membrane formation) were observed in the CLP group. In all groups, alveolar congestion was lowest in the group treated with 3 % NaCl ($p < 0.001$), whereas it was highest in the 0.9 % NaCl treated group ($p < 0.05$). Hemorrhage and thickness of the alveolar wall-hyaline membrane formation scores were decreased in CLP + 3 % NaCl and CLP + HES groups compared to CLP + 0.9 % NaCl and CLP + Ringer Lactate groups ($p < 0.001$). Infiltration-aggregation of leukocytes in air spaces-vessel walls score in the CLP + 3 % NaCl and CLP + Ringer Lactate groups were found to be lower than those in the CLP + 0.9 % NaCl and CLP + HES groups ($p < 0.001$). Perivascular/interstitial edema was also found to be decreased in CLP + 3 % NaCl, CLP + Ringer Lactate and CLP + HES groups compared to the CLP group ($p < 0.001$). Overall, the score of all histological parameters about lung injury decreased substantially in the CLP + 3 % NaCl group relative to other groups (Tab. 1).

Arterial blood gas analysis

When we examine blood gasses, in all groups PaO$_2$ were meaningfully lower than in the control group, however, PaCO$_2$ was meaningfully higher ($p < 0.001$). Likewise, paO$_2$ in the CLP + 3 % NaCl group was significantly higher than in the other groups ($p < 0.01$), moreover, paCO$_2$ was significantly lower in this group ($p < 0.01$) (Tab. 2).

Plasma CRP, IL-6, TNF-α levels

Serum CRP, IL-6 and TNF-α levels of all groups are shown in Table 3. As shown in Figure 2, serum CRP levels were significantly higher in the CLP group compared to the control group ($p < 0.0001$). Among all groups, rats treated with 3 % NaCl had significantly lower CRP levels than other treatment groups ($p < 0.001$).

Plasma levels of TNF-α and IL-6 in all groups are shown in Figure 3 and 4. The plasma levels of TNF-α and IL-6 were significantly higher in the CLP-sepsis group compared with control group ($p < 0.0001$). In the same pattern, there were significant inhibitions in TNF-α and IL-6 levels in the CLP + 3 % NaCl group, compared to the other groups. TNF-α levels were significantly lower in the CLP + 3 % NaCl and CLP + HES groups, while IL-6 levels were significantly lower in the CLP + 3 % NaCl and CLP + 0.9 % NaCl groups compared to the other treatment groups ($p < 0.001$).

Tab. 3. CRP, TNF-α and IL-6 levels.

	Control group	CLP group	CLP and 0.9% NaCl group,	CLP and 3% NaCl group	CLP and Ringer Lactate group	CLP and HES group
CRP (mg/dl)	0.36±0.02	0.79±0.03*	0.57 ±0.02##	0.43 ±0.02##	0.8±0.04	0.63±0.03#
TNF-α (pg/ml)	24.54±11.28	212.38±34.65*	122.38±19.76##	87.60±15.80##	154.67±14.58	107.99±37.52#
IL-6 (pg/ml)	40.53±7.56	524.5±28.4*	228.4±37.8	150.7±25.3##	456.3±48.5	234.0±38.4#

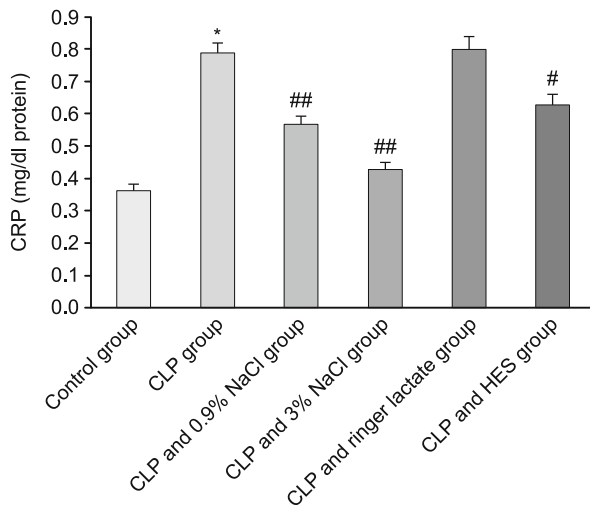* $p < 0.0001$, compared with Control group, # $p < 0.05$, compared with CLP group, ## $p < 0.001$, compared with CLP group

Fig. 2. The effect of volume replacement therapies on serum CRP levels. The results are expressed as mean ± S.E.M. * p < 0.0001, compared with Control group, # p < 0.05, compared with CLP group, ## p < 0.001, compared with CLP group.
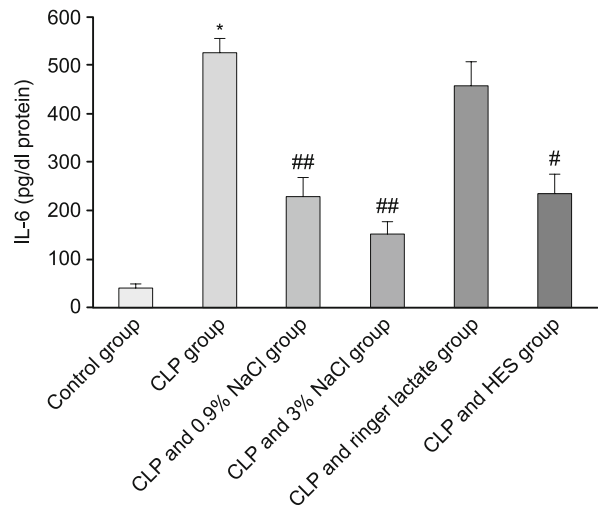

Fig. 4. The effect of volume replacement therapies on serum IL-6 levels. The results are expressed as mean ± S.E.M. * p < 0.0001, compared with Control group, # p < 0.05, compared with CLP group, ## p < 0.001, compared with CLP group.

Fig. 3. The effect of volume replacement therapies on serum TNF-α levels. The results are expressed as mean ± S.E.M. * p < 0.0001, compared with Control group, # p < 0.05, compared with CLP group, ## p < 0.001, compared with CLP group.

Discussion
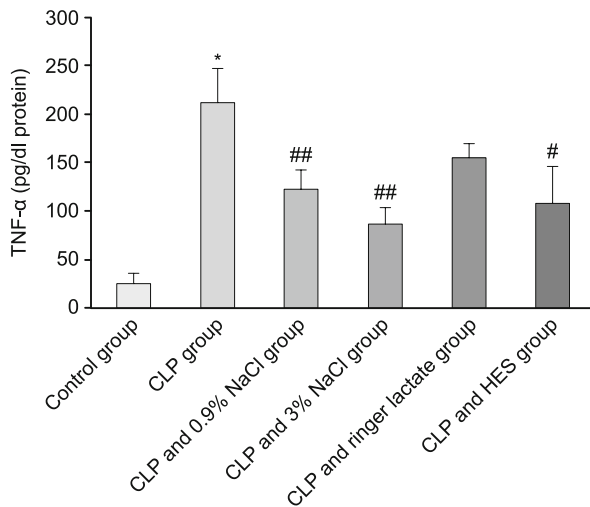
Sepsis is a complicated clinical syndrome with a loud morbidity and mortality rate, with the development of progressive damage to multiple organ systems far from the site of infection (19). First and most severely affected organ is the lung. Therefore, the most common fatal complication of sepsis is acute respiratory distress syndrome (ARDS) (20, 21). As sepsis-induced ARDS is the first step in developing multi-organ dysfunction syndrome in critically ill patients, its prevention is an important therapeutic target.

Mediators that cause inflammatory events in sepsis increase the microvascular permeability and cause leakage in capillaries, causing an increase in interstitial fluid, protein loss and tissue oedema. That is, sepsis is related to intravascular volume deficiency due to venous pooling, vasodilatation and endothelial barrier disruption (22, 23). Due to impaired albumin synthesis and increased loss from the transcapillary membrane, intravascular colloid osmotic pressure (COP) decreases, and the preservation of intravascular volume is impaired. Therefore, sepsis causes organ dysfunction due to insufficient tissue perfusion and oxygenation, resulting in arterial hypotension with reduced cardiac preload and output (24). In this clinical event, fluid resuscitation is the most important thing to restore and maintain the intravascular volume required for microcirculation and maintenance of tissue perfusion (25). As known, severe or extended hypotension is associated with bad clinical outcomes in patients with sepsis, while fast recovery of organ perfusion seems to be correlated with lower mortality rates (26). Nevertheless, there is no consensus on which type of fluid should be used as first-line therapy in resuscitation of septic shock patients.

Many studies have shown that fluid therapy is important in ARDS due to sepsis. However, positive fluid balance has also been shown to harm ARDS patients. Therefore, the key to treatment is to find the balance between pulmonary edema while achieving improvement in tissue perfusion (35). So, we planned this study to investigate which fluid is more effective in lung injury.

Acute respiratory distress syndrome includes microvascular lung damage, including accumulation of leukocyte in the tissue, pulmonary edema and inflammation (23). When we compared pulmonary edema, which suggests a protective effect on the lung, among the groups we used fluid therapy, we found that hypertonic saline was the best preventive measure. The edema interferes with lung function, changes the surfactant and deteriorates the barrier

lesions of the alveolocapillary. Any effort to decrease edema could therefore have beneficial effects on respiratory function. Similar results have been found in other studies (27).

The inflammatory mechanism introduced during ARDS is characterized by the release of inflammatory mediators and the inflow of leukocytes in the pulmonary tissue (28). In our study, we also observed an increase in leukocyte infiltration in the untreated CLP group. We found that leukocyte infiltration in the lungs of groups treated with 0.9 % NaCl and HES could not be adequately prevented, but treatment with hypertonic saline significantly reduced the content of leukocytes. Supporting this, Oliveira et al suggests that the most interesting beneficial effect of hypertonic saline is the reduction of SIRS and attenuated MODS, especially in sepsis (29).

In consideration of perfusion, there was a study in the rat sepsis model caused by peritonitis (CASP) which determined that administration of the hydroxyethyl starch (HES) solutions provided no additional benefit on improvement of microcirculation in comparison to crystalloid solutions (30). Similarly, with regard to organ failure, it has been shown that albumin had no advantage over Ringer's Lactate (RL) on efficacy in reducing ventilator induced lung injury in an endotoxic rat model of sepsis (31). Nonetheless, there was also another study in rat cecal ligation and puncture (CLP) model of sepsis which showed that unbalanced crystalloid solution (0.9 % NaCl) was more effective on the development of acute kidney injury (AKI) and survival time when compared to a balanced crystalloid (Plasma-Lyte) (32). In another study in an endotoxic rat sepsis model it has been reported that synthetic colloid solution Hextend reduced mortality and lessened metabolic acidosis when compared to resuscitation with the saline or RL (33).

Unlike complicated findings from small-animal models, experiments in large animal models usually support the administration of colloid solutions in sepsis. Qui, et al. studied the effects of crystalloids (0.9 % NaCl and 7.5 % NaCl) or colloid (HES) solutions, on levels of vascular endothelial growth factor (VEGF), capillary permeability, and development of pulmonary edema in a sepsis dog model (2). Shown in studies, HES was more effective than crystalloid solutions in reducing tissue expression of VEGF, capillary permeability, and lung edema levels (34). However, there was another study performed in a sheep model of peritonitis induced sepsis which showed that even though the albumin and HES could improve the cardiac output, delivery of oxygen and reduce the blood lactate levels, there was no change in survival when compared with the administration of the crystalloid, RL (35).

In addition to the importance of clinical results associated with the amount and form of fluid resuscitation in septic care, the fundamental mechanisms to mediate these responses must also be examined. Recently, it has been shown that Nitric Oxide (NO) had a main role in sepsis pathophysiology. As it is known, the endothelium displays a vasodilatory response to fluid loading in order to tolerate increased blood flow and endothelial shear stress. In a hamster model of lipopolysaccharide (LPS)-induced sepsis, it has been demonstrated that enhancing or ameliorating NO bioavail-

ability throughout sepsis may improve arteriole dilation, functional capillary density, and survival albeit reducing leukocyte endothelial interactions (2, 36). Vasopressin 1 receptor (V1R) is another player that has an important role in volume resuscitation therapy. It has been determined that in an endotoxic rat model, volume resuscitation can cause vasopressin release to enable the necessary V1R during the periods of low vasopressin secretion for an increased blood pressure in response to the fluids (37). The same pathway was also active and served a function after volume resuscitation in the CLP rat model of severe sepsis (38).

With a regard to inflammatory mechanisms, it has been shown that circulating TNF-α levels were substantially decreased in volume resuscitated animals compared to controls in the endotoxic rat sepsis model (2). The reduced TNF-α correlated with decreased total peripheral resistance (TPR), high cardiac beats, and higher survival rate (39). C-reactive protein (CRP) is an acutephase protein, which increase in blood rapidly in response to infection, trauma, ischemia, burns, and other inflammatory conditions (40). Because of these results, plasma CRP levels have been evaluated for a long time as an important biomarker for evaluating systemic inflammation (41). CRP levels have been shown to have prognostic/diagnostic significance in a great range of diseases, with the inclusion of sepsis, pneumonia, coronary artery disease, stroke and diabetes (42–44). Some studies indicate that CRP may be an indication of progressive organ damage (45–47). ARDS is an inflammatory process and many studies have centered on determining the function of inflammatory plasma mediators in this disease and how they correlate with phenotypes and clinical outcomes (18, 48, 49). While literature studies associating CRP levels with prognoses in other diseases have been performed, there is little knowledge of the CRP function in ARDS and ALI patients. In our study, we also found a significant decrease in CRP, TNF-α and IL-6 levels in the CLP + 3 % NaCl group when compared with the other groups. Therefore, this may be the first data about CRP and proinflammatory cytokine levels in sepsis-induced ARDS/ALI and since inflammatory processes have important role in sepsis, the effects of volume resuscitation on CRP levels and its underlying mechanisms need to be investigated in detail.

In our study, we showed that hypertonic saline was the most effective fluid for volume resuscitation. It reduced the lung injury parameters histologically, improved functional parameters in analysis of arterial blood gas and also had some anti-inflammatory effects with regard to CRP, TNF-α and IL-6 levels.

Conclusion

We demonstrate that hypertonic saline 3 % NaCl-treatment significantly mitigates lung injury, with quantifiable suppression of injury parameters on lung histochemistry, increase in paO₂ and reduction in paCO₂ in the arterial blood gas analysis, and also decrease in CRP, TNF-α and IL-6 levels. We also found that colloid solution HES can be more effective when compared with other crystalloids, 0.9 % NaCl and Ringer Lactate. Our findings replicate earlier published findings which demonstrated that hypertonic saline could ameliorate the ARDS/ALI in another rat model

of sepsis and add to the body of evidence that hypertonic saline may serve as a first line therapy. Further studies are needed before these fluids can be suggested as a first line treatment and also for clarification of the underlying mechanisms of these different resuscitation fluids in sepsis-related ARDS/ALI.

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