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Original Article

Effects of Emulsified Sevoflurane and Ulinastatin on Liver and Lung Injury Induced by Bile Duct Ligation in Rats

Cai-Yang Chen*, Ming Zhu*, Long Wang, Fei-Xiang Wu, Li-Qun Yang, and Wei-Feng Yu

ABSTRACT

Backgroud: To investigate the effects and their mechanisms of emulsified sevoflurane and ulinastatin on liver and lung injury induced by obstructive jaundice in rats.

Methods: For the in vivo study, male Sprague-Dawley rats were randomized into two parts. Part 1: Sham group, bile duct ligation (BDL) group, BDL and lipid vehicle infusion (BDL + V) group, BDL + ulinastatin (BDL + U) group, BDL + emulsified sevoflurane (BDL + E) group; Liver and lung function was examined by the concentrations of Total Bilirubin (TBIL), Alanine Transaminase (ALT), and lung arterial blood gas analysis. Liver and lung damage was estimated histologically with haematoxylin and eosin (HE) staining in liver and lung samples. F4/80, the molecular marker of activated macrophages, was assessed by immunohistochemistry (IHC). Part 2: Sham + Gadolinium chloride ($GdCl_3$)group, BDL + $GdCl_3$ group, BDL + U + $GdCl_3$ group, BDL + E + $GdCl_3$ group. After the deletion of macrophages, liver and lung examinations were evaluated histologically with HE staining in $GdCl_3$ treated rats. For the in vitro study, the RAW264.7 cell line was stimulated by the different treatments, then terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL staining), Hoechst 33342 staining and the expressions of apoptotic related genes were evaluated to further determine the protective effects of ulinastatin and emulsified sevoflurane.

Results: Emulsified sevoflurane and ulinastatin therapies alleviated cholestatic liver and lung damage, inhibited the activation of macrophages in the liver and lung tissues, and improved the liver and lung functions. And they were also found to inhibit apoptosis pathways.

Conclusions: Emulsified sevoflurane and ulinastatin treatments were proved to ameliorate BDL-induced cholestatic liver and lung injuries, which were related to the inactivation of the macrophages and the regulation of apoptosis pathways. The above-mentioned mechanisms show new promising approach for the multi-organs injuries in the perioperative management.

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Obstructive jaundice is a common clinical disorder presenting in the gallbladder carcinoma, cholangiocarcinoma, primary sclerosing cholangitis, gallstones, which is also associated with fetal complications such as hepatopulmonary syndrome (1), renal dysfunction (2), and multiple organ failure syndrome (3). Hepatopulmonary syndrome (HPS) is defined as an arterial oxygenation disorder induced by the dysfunction of liver and lung. And the bile duct ligation for rat is an ideal model for the studies on the HPS by obstructive jaundice. Current articles (4- 7) have proved that inflammatory response and apoptosis play vital roles on the progression of obstructive jaundice. Previous studies (8) have clarified that tumor necrosis factor-related apoptosis worsened cholestatic hepatotoxicity. Tiao MM et al have also observed that expression of the pro- apoptotic gene Bax was significantly increased after BDL (9) and micro RNA-29a protected against cholestatic liver injury by inhibiting the extrinsic apoptosis pathway (10).

In clinical anesthesia settings, as common anesthetic and anti- inflammatory drugs, sevoflurane and ulinastatin have often been used together during the surgery. Sevoflurane is a widely used clinical inhalation anesthesic. Increasing evidences have proved that sevoflurane preconditioning could protect against multiple organ injury, such as lung, liver, and brain (11, 12). Emulsified sevoflurane has been synthetized that comprise emulsification of sevoflurane into a lipid vehicle and simplifies its intravenous application in clinic. Ulinastatin, urinary trypsin inhibitor (UTI), is an acid- resistant Kunitz- type protease inhibitor. Recent studies have shown that UTI may protect against the production of inflammatory mediators (13- 15). More researches have suggested that UTI may protect against liver and lung injury (16, 17). According to the above mentioned studies, both emulsified sevoflurane and ulinastatin have some good effects on anti inflammation of liver and lung.

Since the mechanisms of multi organs injuries induced by obstructive jaundice are so intricated, an increasing number of researches have concentrated on the treatments for diminishing the complications. However, it is still unknown whether both of emulsified sevoflurane and ulinastatin alleviate obstructive jaundice related hepatopulmonary syndrome and inhibit the activation of apoptotic pathway. Therefore, we show here that this research intends to estimate cholestatic hepatopulmonary syndrome and the mechanisms of emulsified sevoflurane and ulinastatin in attenuating the syndrome. It is anticipated that more convincing evidences on the treatment of obstructive jaundice are gained, and further perioperative applications of emulsified sevoflurane and ulinastatin against the complications will be illuminated.

MATERIALS AND METHODS

All experiments were approved by the Animal Care and Use Committee of the Second Military Medical University (Shanghai, China) and were conducted according to Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. A total of sixty healthy male Sprague- Dawley rats of clean grade, weighting 200-250 g, were provided by the Laboratory Animal Research Center of the Second military medical university. These rats were maintained on standard laboratory conditions.

Animal Grouping

Rats were randomly divided into Sham operation group, BDL (bile duct ligation) group, BDL + U (ulinastatin) group, BDL + E (emulsified sevoflurane) group, BDL + V (lipid vehicle) group. In Sham operation group, rats were assigned to laparotomy and dissection of the bile duct but not ligation. In BDL groups, rats were randomly assigned to receive intraperitoneally 0.9% saline, ulinastatin, emulsified sevoflurane (3.5%) and lipid vehicle, in different 4 groups. In BDL + U and BDL + E groups, ulinastatin and emulsified sevoflurane were injected intraperitoneally 100,000 U/kg and 1 ml/kg, respectively, to the rats and the same dose injection was maintained every day for 21 days. In BDL and BDL + V group, equal volume of normal saline solution and lipid vehicle were applied, respectively.

Subsequently, in order to observe the roles of macrophages and then delete macrophages from the liver and lung, more 4 groups of rats were used to be injected intravenously with a 100ul volume of $GdCl_3$ (10 mg/kg body weight, through the tail vein, 24 h before the

procedures, twice a week). Rats were randomly divided into sham + $GdCl_3$ group, BDL + $GdCl_3$ group, BDL + U + $GdCl_3$ group, BDL + E + $GdCl_3$ group.

Reagents

Emulsified sevoflurane was obtained from Prof. Jin Liu, the Laboratory of Anesthesiology and Critical Care Medicine, West China Hospital, Sichuan University (Chengdu, China); UTI was purchased from Tianpu Biochemical Pharmaceutical Co. Ltd. (China); F4/80 antibody was obtained from Santa Cruz Biotechnology, Inc (USA); TUNEL assay kit was purchased from Takara Bio Inc (Japan). Hochest kit was purchased from Beyotime (China).

Surgical Procedures of Bile Duct Ligation

A common model of BDL and sham procedures were used exactly as previously described (18). The rats were anesthetized with pentobarbital (50 mg/kg body weight) by intraperitoneal injection. After the anesthesia, laparotomy was performed through a midline abdominal incision. The common bile duct was dissociated and exposed, double-ligatedand cut. Then the abdomen was closed with a layered suture. Rats were sacrificed at 21 days after the surgical procedures. Blood samples, liver and lung biopsies were collected for analysis.

Histological Procedure

Organ tissues from liver and lung were fixed in 10% formaldehyde for 24 h and embedded in paraffin. The dissected sections were cut and stained with hematoxylin-eosin (H&E) for light microscopy. The histological examination was performed and analyzed by skilled histologists, blinded for all groups.

Immunohistochemical Assay

Immunohistochemical staining was conducted by the procedures mentioned previously. The dissected sections were cut and stained for F4/80. Briefly, paraffin embedded lung and liver sections were deparaffinized in xylene and rehydrated in different concentrations of ethanol. These sections were treated with citrate buffer to retrieve antigens, 10% fetal calf serum to block non-specific binding. The primary anti-macrophage F4/80 antibody were applied at 4℃ all night.

Biochemical Measurements

The right carotid artery and the left jugular vein were cannulated for arterial blood-gas analysis and blood sampling collection, respectively. 3-5 milliliter of blood was drawn from the left jugular vein for liver function, while 1 ml of blood was drawn from the right carotid artery for blood gas analyze. After the centrifugation of blood, the supernatants were deposited at -80℃ for the later analysis. Serum alanine aminotransferase (ALT) was performed for samples by an autobiochemistry analyzer (Hitachi 7600-020, Tokyo, Japan). Blood gas analysis was determined by an auto blood-gas analyzer (GEM Premier 3000, Instrumentation Laboratory, USA).

Cell Lines and Cell Culture

Raw 264.7 cells were obtained from the Cell Research Institute of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in RPMI 1640 medium supplemented with 10% serum of rats from sham group or BDL group at 37℃ in a humidified incubator containing 5% CO_2.

Emulsified sevoflurane and ulinastatin were dissolved as described previously. In brief, ulinastatin were freshly diluted in RPMI 1640 medium. Thenceforth, 0.2% emulsified sevoflurane and 1000U/L ulinastatin were added to the medium with 10% serum of rats from BDL group, respectively. According to the experimental design (Figure 1), equal volumes of saline and lipid vehicle were added to the relevant groups.

Real-Time Polymerase Chain Reaction Analysis of Bax and Bcl-2 mRNA Expression in RAW 264.7 Cells

The mRNA expression of pro-apoptotic Bax and anti-apoptotic Bcl-2 was conducted by previous procedures using RAW 264.7 cells, in the existence of RPMI 1640 medium with 10% serum of rats from sham group or BDL group at 37℃. Total RNA was isolated from RAW 264.7 cells by using Trizol reagent (Invitrogen, Life Technologies) and reversely transcribed into the complementary DNA (cDNA), according to the previous instructions (20). The cDNA template was mixed with the SYBR Green PCR Master Mix.

Real-time PCR was conducted with the Applied BioSystems 7900. The primers, designed by Primer Premier 5.0, were as follows: Bax forward: 5'- ATCCAGGATCGAGCAGGGCG- 3', and reverse: 5'- ACTCCCTCAGCTCTTGGTG- 3'; β- actin, as an internal reference gene, forward: 5'- GCGAGAAGATGACCCAGATCAT- 3', and reverse: 5'-GCTCAGGAGGAGCAATGATCTT - 3'. Every sample was measured by 2 times. The relative mRNA expressions of Bax and Bcl- 2 were measured by the $2^{-\Delta\Delta CT}$ method.

Apoptosis Detection

For apoptosis analysis, cells were seeded into 6-well plates with 5×10^5 cells/well and incubated overnight followed by treatment. The TUNEL staining was performed according to the instruction. Apoptotic cells in the liver were detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining using In Situ Apoptosis Detection Kit (Calbiochem), and the nucleus was counter stained with methyl green.

The Hoechst 33342 staining was performed according to the instruction. After incubated by DNA-specific dye Hoechst 33342 with the proper concentrationof 5 μg/ml, nuclear DNA of RAW 264.7 cells were observed by the microscope with the blue fluorescence filter.

Statistical Analysis

All the results in the figures from the research were showed as mean ± standard deviation (SD). Data was analyzed by statistical software: SPSS 17.0. All variables were tested for the distribution and all of them were normally distributed. Statistical differences for above indexes were measured by one- way analyses of variance (ANOVA) followed by Tukey HSD post-hoc test. Two- tailed P- values less than 0.05 were considered statistically significant.

RESULTS

Effect of Emulsified Sevoflurane and Ulinastatin on Attenuating BDL Induced Liver Damage of Rats

Liver Function after BDL

BDL group indicated biochemical measurements and histological examinations of liver injury as reflected by higher plasma concentrations of ALT and HE staining, and lung dysfunction as reflected by blood gas analyses and HE staining. However, after BDL 21d, both mouse strains indicated protective effects after the injection of emulsified sevoflurane and ulinastatin(both $P<0.05$ versus BDL group). The concentrations of serum ALT in BDL group were sharply higher than the control and the treatment groups. Compared to other groups, the serum ALT level was increased in BDL group. The variances between ES group and UTI group were not significant. Compared with sham group, the BDL, BDL + U, BDL + E and BDL + V groups had significantly higher serum total bilirubin levels ($P<0.05$). But there were no significant difference between the above groups ($P>0.05$) . Compared with sham group, the BDL, BDL + U, BDL + E and BDL + V groups had significantly higher serum ALT levels. Compared with BDL and BDL + V group, BDL + U, BDL + E had significantly lower serum ALT levels ($P<0.05$) (Figure 2).

Histopathological Changes of the Liver

Liver histology was normal in sham group. In contrast, histopathological examination indicated that the liver tissues in the saline and lipid vehicle groups (BDL, BDL + V) were severely damaged 21 days after bile duct ligation, as represented by marked infiltration of leukocytes and partial destruction of the liver architecture, while only moderate liver inflammatory cell infiltration were seen in emulsified sevoflurane and ulinastatin treatment groups (BDL + U, BDL + E), indicating that liver damage induced by BDL was attenuated by emulsified sevoflurane and ulinastatin groups (Figure 2).

Effect of Emulsified Sevoflurane and Ulinastatin on Attenuating BDL Induced Lung Damage of Rats

Arterial Blood Gas Analysis

Compared with sham, BDL + U and BDL + E groups, the BDL and BDL + V groups had significantly lower pH, PaO_2 and higher A- aDO_2 ($P<0.05$). Treatment with emulsified sevoflurane and ulinastatin improved pulmonary function, in accompany with higher pH, PaO_2 and lower A- aDO_2, while $PaCO_2$ in BDL and BDL + V groups were lower than those in sham, BDL + U

and BDL + E groups, but there were no significant difference between them($P > 0.05$, Figure 3). The BDL group had markedly lower PaO_2 and higher $PaCO_2$ than the control group. Compared with BDL group, the BDL + UTI and BDL + ES group had higher PaO_2 and lower $PaCO_2$. The BDL + V group had remarkably lower PaO_2 and higher $PaCO_2$ than the BDL + ES group, but there is no significant difference between BDL group and BDL + V group (Figure 3).

Lung Histopathology after BDL

Lung histopathology and blood gas analyze was aggravated in BDL group as compared with sham group. However, there was significant difference between emulsified sevoflurane and ulinastatin group. The protective effects of emulsified sevoflurane and ulinastatin treatments on the morphologic changes of the lungs in rats with BDL are shown in Figure 3. Histopathological changes of the lung. Sham group: No morphologic alternation was observed. BDL group: the inflammatory process was observed as represented by infiltration of leukocytes into interstitial and alveolar spaces, edema and partial destruction. Blind analysis was conducted on all samples to evaluate pulmonary structure, tissue edema formation and infiltration of the inflammatory cells. Pulmonary histology was normal in sham group. In contrast, histopathological examination indicated that the lung tissues in the saline and fat vehicle groups (BDL, BDL + V) were severely damaged 21 days after bile duct ligation, as represented by marked infiltration of leukocytes into interstitial and alveolar spaces, edema and partial destruction of the pulmonary architecture, while only moderate lung edema, inflammatory cell infiltration and thickening of the alveolar wall were seen in emulsified sevoflurane and ulinastatin treatment groups(BDL + U, BDL + E), indicating that lung damage induced by BDL was attenuated by emulsified sevoflurane and ulinastatin groups (Figure 3).

Effect of Emulsified Sevoflurane and Ulinastatin on Attenuating BDL Induced the Activations of Macrophages

The accumulation and activation of macrophages in the liver and lung proved to be a crucial promoter of liver and lung injury. BDL challenge led to the activation of macrophages in the liver and lung. Compared with sham group, increased expression of F4/80 was observed in BDL group. The expression of F4/80 in liver and lung was significantly attenuated by emulsified sevoflurane and ulinastatin treatment. These changes were no difference between emulsified sevoflurane and ulinastatin group (Figure 4).

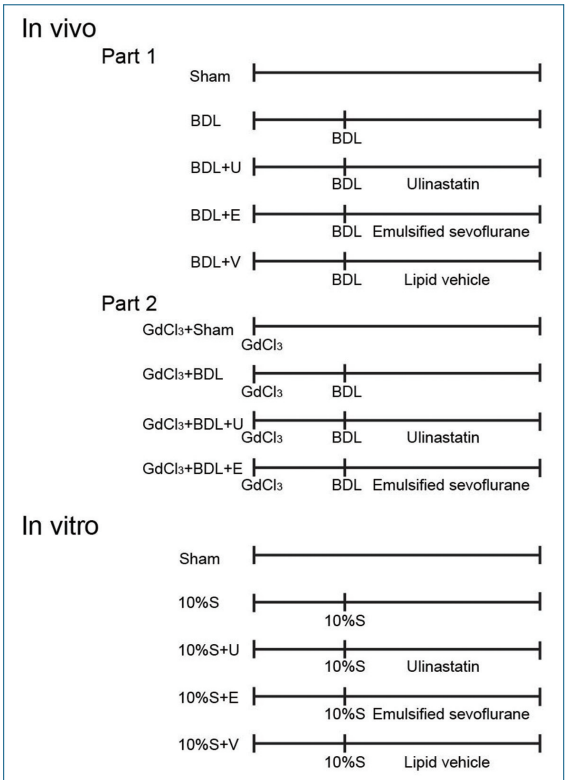

Figure 1. An Illustration of Experimental Design

Effect of Emulsified Sevoflurane and Ulinastatin on Attenuating BDL Induced Liver and Lung Damage of Rats after Inhibiting the Macropages

Inhibiting macrophages with gadolinium chloride ($GdCl_3$) partly prevented the injury of liver and lung in BDL groups. Compared with $GdCl_3$ + Sham group, the administration of $GdCl_3$ resulted in the significant attenuated inflammatory cell infiltration and tissue edema formation of liver and lung in the histological examination in $GdCl_3$ + BDL, $GdCl_3$ + BDL + U, $GdCl_3$ + BDL + E groups. These changes were no difference between emulsified sevoflurane and ulinastatin group (Figure 5).

Apoptotic Detection of Raw 264.7 Cells

Real- time PCR was conducted on RAW 264.7

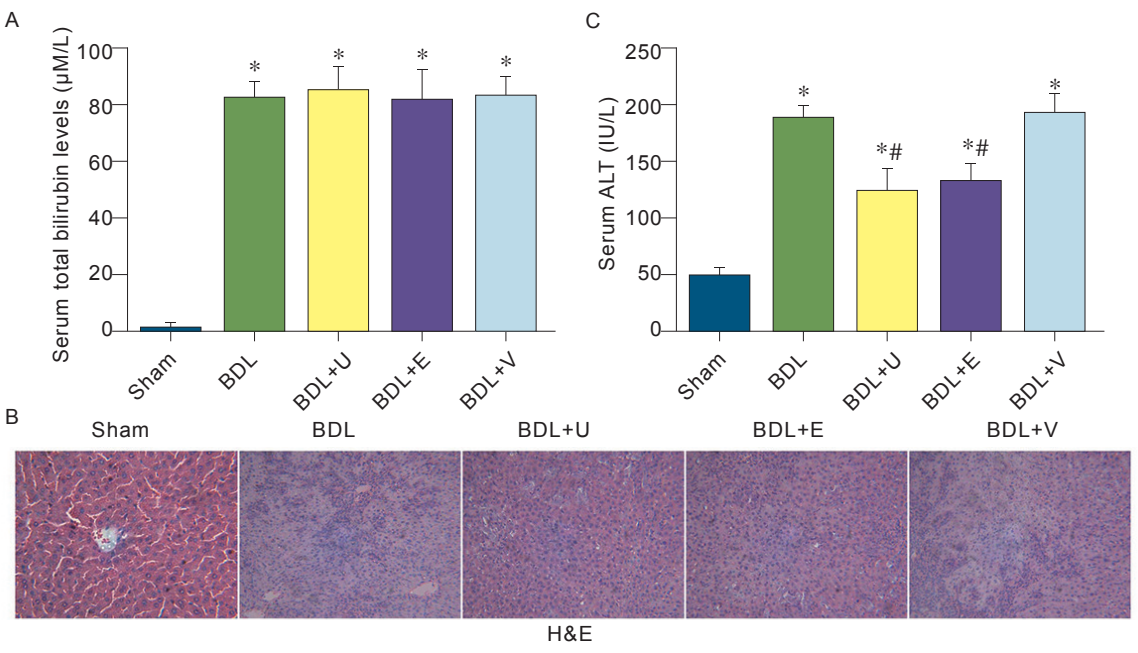

Figure 2. Biochemical Measurements and Histological Examinations on Liver Tissue Specimens at 21 Days after BDL Procedure (N=6).
*P<0.05 vs. Sham group; #P<0.05 vs. BDL and BDL+ V group.

cell lines to detect whether apoptosis pathway is activated after the existence of bilirubin. RT-PCR analysis of Bax and Bcl-2 mRNA expression in the Raw 264.7 cells after treatment with 10% serum of BDL rats in 1640 medium. Real-time PCR analysis indicated that Bax mRNA expression was over-expressed in the treatment groups. Compared with 10% S and 10% S + V groups, emulsified sevoflurane and ulinastatin treatments decreased that Bax mRNA expression and increased Bcl-2 mRNA expression significantly. Bax and Bcl-2 mRNA expression were up-regulated markedky in 10%S, 10%S+U, 10%S+E, 10%S+V groups(Figure 6).

Apoptotic Detection of Raw 264.7 cells by Terminal Deoxynucleotide Transferase-mediated Deoxyuridine Triphosphate Nick-end Labeling and hoechst 33342. These data show that apoptosis pathway is activated in the macrophage after the treatment (Figure 6).

DISCUSSION

Obstructive jaundice is always accompanied with high perioperative mortality (19). Pro-inflammatory response has been regarded as a major pathogenesis of severe complications of patients with obstructive jaundice (20). The lung and liver are almost considered to be the most vulnerable organs in patients with systemic inflammatory response syndrome (21). The concrete mechanisms on perioperative hepatopulmonary syndrome and multiple organ failure syndrome in patients with obstructive jaundice are still unclear. It is well-known that patients with obstructive jaundice are more vulnerable to a rapidly fatal infection, but there was little information on anesthesia-related therapeutical methods. In the present study, we designedthis project to investigate whether emulsified sevoflurane and ulinastatin mightattenuate the injury in vivo, and whether the protection might be related to the apoptosis pathway in vitro.

In the present study, bilirubin-treated RAW 264.7 cells showed the over-expression of an anti-apoptotic gene Bcl-2 and pro-apoptotic gene Bax. These results were in agreement with TUNEL staining which indicated a high increase in the number of positive apoptotic body in the bilirubin-treated group. Also, it is proved that obstructive jaundice induced the over-expression of Bax and caspase-3 which led to the

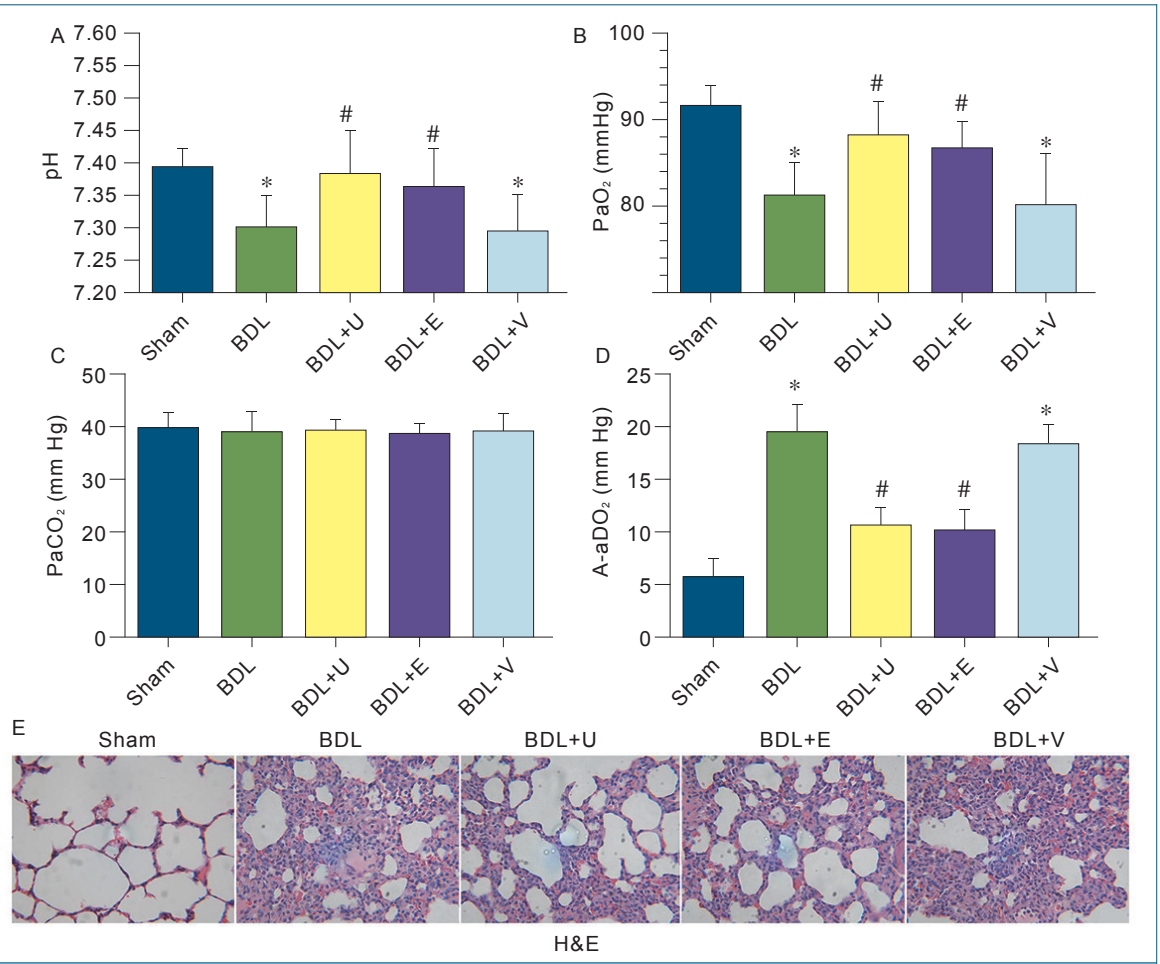

Figure 3. Arterial Blood Gas Analysis and Histological Examinations on Lung Tissue Specimens at 21 Days after BDL Procedure (N=6).
*P<0.05 vs. Sham group; #P<0.05 vs. BDL and BDL+ V group.

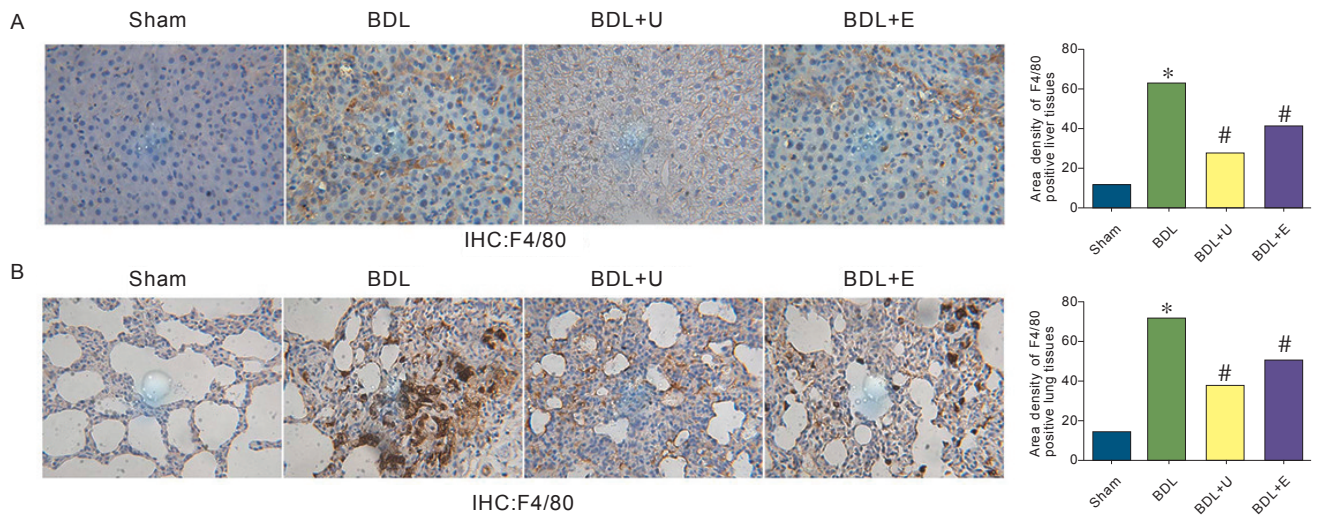

Figure 4. Changes in the Activations of Liver and Lung Macrophages at 21 Days after BDL Procedure (N=6).
*P<0.05 vs. Sham group; #P<0.05 vs. BDL group.

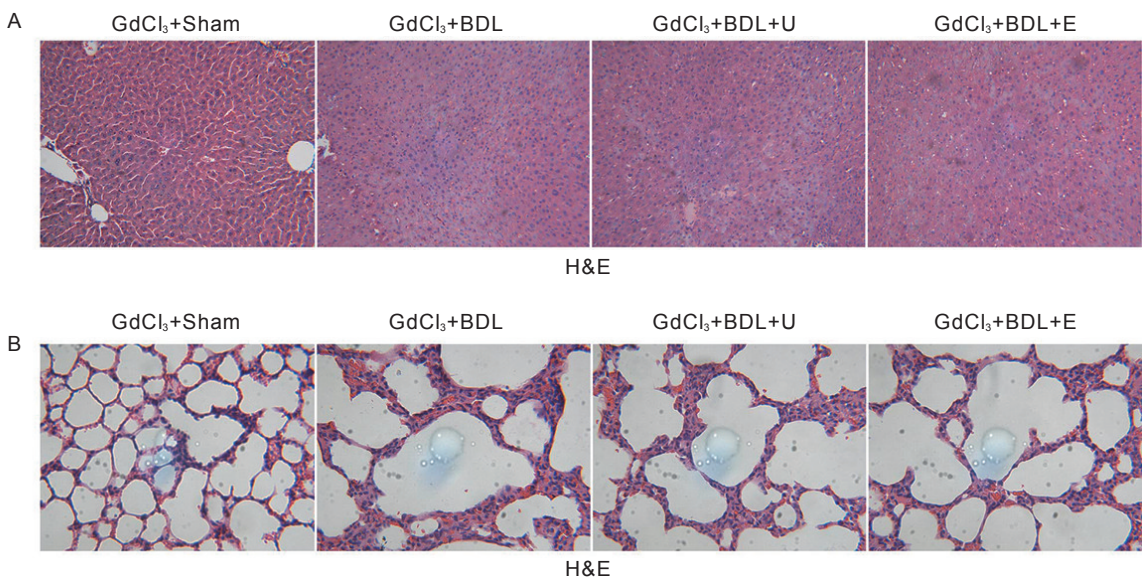

Figure 5. Histological Examinations on Liver and Lung Tissue Specimens after Inhibiting the Macrophages (N=6).

apoptosis of multi organs in BDL rats. Furthermore, there is evidence that BDL-induced obstructive jaundice modifies the expression of anti-apoptotic Bcl-2 and pro-apoptotic Bax. The present study showed the treatment of obstructive jaundice related hepatopulmonary with ulinastatin significantly decreased the plasma ALT level and liver lesion.

However, histological examinations for liver and lung after 21 days were not performed. Blocking the macrophages of the lung and liver with the administration of $GdCl_3$ did not totally inhibit the damage in BDL group, when compared with sham group. The activation of macrophages was assumed to play an important part in the initiation of inflammatory response, since blockade of macrophages alleviate the progression of lesions.

Activated macrophages, as an important antigen presenting cells, are one of the vital initiation elements in the multi organs damage with obstructive jaundice. In our study, we showed that alleviated liver and lung injuries are involved in BDL mice by the administration of the gadolinium chloride, a kind of specific macrophages blocking agents. Many researches have proved that tumour necrosis factor alpha (TNFα) is mainly produced by macrophages in different kinds of hepatic diseases (22, 23) .

The mechanisms based on emulsified sevoflurane and ulinastatin-induced liver and lung protection may be multifactorial. Because of the existence of the free radicals, antioxidant and apoptotic enzymes may increase duringthe inflammatory response. Compared to the control group, the expression of apoptotic and anti-apoptotic genes increased. Apoptosis is a pathway that has been implicated in the progression of various inflammatory reponses. The research findings compliance with the consequence that hepatopulmonary syndrome induced by obstructive jaundice was partly mediated by the activation of macrophage and inhibition of apoptosis pathway.

In conclusion, obstructive jaundice has been generated in mice by bile duct ligation model. In this study, this model is accompanied with multi-organs damage, including liver and lung lesions. In addition, the liver and lung lesions can be reversed by blocking the activation of macrophages and decreased by the administration of emulsified sevoflurane and ulinastatin from these organs. However, renal histological examination and renal function related measurements are absent in this study. Therefore, it is still unclear exactly that the protections of emulsified sevoflurane and ulinastatin with systemic multi-organs in patients with obstructive

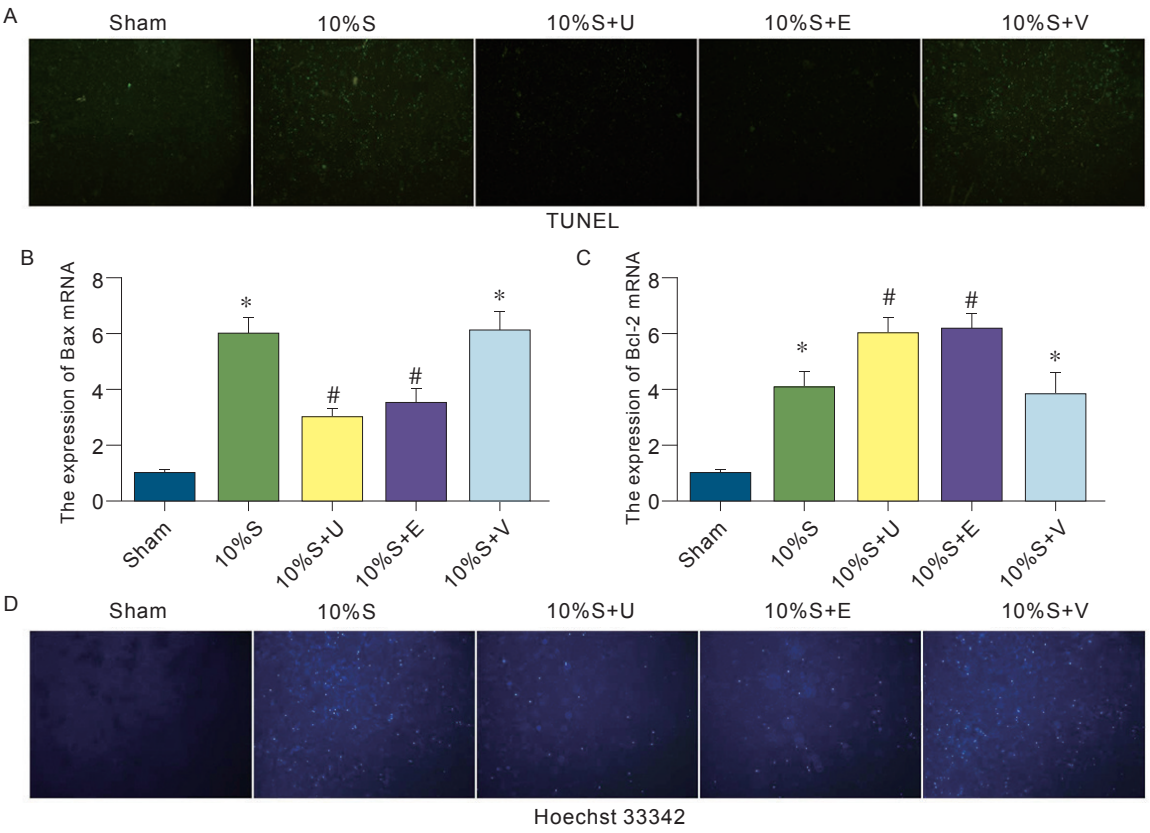

Figure 6. Apoptotic Detections in the Raw 264.7 cells.
*P<0.05 vs. Sham group; #P<0.05 vs. 10%S and 10%S+V group.

jaundice. And the theory on the protections of emulsified sevoflurane and ulinastatin with obstructive jaundice is not still completely clear. It may partly be relevant to the activation of macrophage and apoptosis pathway, and inflammatory and necrosis pathway are also involoved in that process.

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All authors have no other potential conflicts of interest for this study to declare.

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