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论文题目 **High-mobility group box 1 and scavenger receptor A: novel applications in the management of patients with cholangitis**

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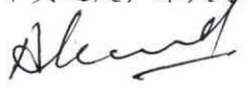
学位论文作者签名：  日期：2016.3.17

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To Father and Mother

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ABBREVIATIONS

Abbreviation	Full form
AOC	Acute Obstructive Cholangitis
AOSC	Acute Obstructive Suppurative Cholangitis
BDL	Bile duct ligation group
ELISA	Enzyme-linked immunosorbent assay
HMGB1	High Mobility Group Box 1
IL-10	Interleukin -10
IHC	Immunohistochemistry
KCs	Kupffer cells
LDL	Low-density lipoprotein
MCP	Masculine Cell Population
MODS	Multi-organ dysfunction syndrome
PBMCs	Peripheral blood mononuclear cells
RAGE	Receptor for Advanced Glycation Endproduct
RT-PCR	Real Time Polymerase Chain Reaction
SO	Sham operation group
SRA	Scavenger receptor A
TLRs	Toll-like receptors
TNF - alpha	Tumor Necrosis Factor – alpha

High-mobility group box 1 and scavenger receptor A: novel applications in the management of patients with cholangitis

Abstract

Background:

Cholangitis is the inflammation of biliary ducts which usually begins extra-hepatic and easily spreads intra-hepatic, causing bacteremia. Acute obstruction of biliary tract with the presence of pus is severe form of cholangitis know as Acute Obstructive Suppurative Cholangitis (AOSC) which has very high mortality. It is experienced that the Charcot's triad and Reynold pentad are difficult to use in clinical practice for the management of cholangitis and recently published "Updated Tokyo Guidelines for management of cholangitis and cholecystitis in 2013 is lack of evaluation and revision.

High Mobility Group Box 1 (HMGB1) is a proinflammatory cytokine which is found to play an active role during the pathogenesis of inflammatory processes. In response to infection, HMGB1 acts as an important proinflammatory cytokine which may lead in the damage of cells and tissues. This process may continue to metabolic acidosis, multi-organ dysfunction syndrome (MODS) and sepsis. Recent studies have reported associations between HMGB1 and inflammation. But the correlation between HMGB1 and AOSC pathogenesis is not yet fully understood. We detect whether HMGB1 is involved in the pathogenesis of AOSC.

Scavenger receptor A (SRA) is a transmembrane glycoprotein and mainly distributed in hepatic Kupffer cells. It is SRA that relates to defensive reaction which mediates macrophage clearing and inactivating endotoxin. The relation between SRA and liver tissue during inflammation is not clearly described in the literatures. We intend to commence from AOSC and establish animal model of Wistar rats cholangitis so that we might investigate the expression of SRA and their relations to endotoxin, TNF-α, IL-6 and endotoxic hepatic injury in AOSC in order to uncover the mechanism of the conversion of Kupffer cells from immune defensive cells to inflammatory response cells during AOSC from histology, cell, molecule and gene, which may provide evidence for the establishment of new therapeutic regimen for AOSC.

Purpose:

This paper include two parts, (I) relationship between HMGB1 and Cholangitis patients and (II) relationship between SRA and mouse model of cholangitis to provide evidence for the establishment of new management regimen for Cholangitis.

Methods: We collected samples of peripheral blood from 23 patients with AOSC and 23 healthy volunteers who served as normal controls. All were tested for the HMGB1 mRNA, the HMGB1 protein, Tumor Necrosis Factor - alpha (TNF - alpha) and Interleukin -10 (IL-10). HMGB1 mRNA was tested using RT-PCR.

HMGB1 Protein was tested using Western blot. TNF-alpha and IL-10 were tested using ELISA.

Also, Mouse model of AOC were reproduced by ligating choledochus and injecting *Escherichia coli* O_{111}B4 into it. The concentration of plasma endotoxins were assayed by litmus test. The level of tumor necrosis factor-alpha (TNF-alpha) and Interleukin-6 (IL-6) in plasma were determined with enzyme-linked immunosorbent assay (ELISA). The expression of SRA protein in liver tissue was assayed by immunohistochemistry, and the expression of SRA mRNA in liver tissue were detected via light microscope.

Results: The expression of HMGB1 mRNA and HMGB1 Proteins were higher in AOSC group than that of normal controls ($P<0.01$) and it gradually decreased to normal level after the treatment of the disease ($P<0.01$). The content of TNF-alpha and IL-10 in peripheral blood of AOSC patients was significantly higher than the content of normal controls ($P<0.01$) and they decreased to normal level after the necessary treatment ($P<0.01$).

The plasma endotoxins concentrations in AOC were progressively increased with the time prolonged. With increasing endotoxin, the level of TNF-alpha and IL-6 were also markedly increased. The SRA protein and mRNA expression were obviously decreased with prolonged experimental time. There were significant difference among experimental groups and control groups ($P<0.01$). Liver histopathological study showed that the hepatic injury was gradually aggravated

with the time prolonged. Massive inflammatory cells infiltration and large-area hepatocytes degeneration and necrosis were found in later period.

Conclusions: The level of HMGB1 mRNA and HMGB1 Protein are elevated in patients with AOSC which may have important role in the inflammation of bile duct and seems to be associated with the development of sepsis suggesting its importance in the management of AOSC-induced sepsis.

The expression of SRA in Kupffer cells gradually decreases with progressively increasing plasma endotoxin levels in AOC. Its ability of clearing and inactivating LPS correspondingly descends. In the meantime, endotoxin strengthen activating Kupffer cells. Hence, it might be one of the important mechanism for the conversion of Kupffer cells from immune defensive cells to inflammatory response cells. We found, the decreased expression of SRA in Kupffer cells is responsible for endotoxin hepatic injury in AOC.

Key Words: Acute Obstructive Cholangitis; HMGB1; Interleukin-10; TNF-alpha, Sepsis; Inflammation; Scavenger Receptor A; Endotoxemia; Cytokine, Endotoxin Hepatic Injury.

(I) Role of High Mobility Group Box 1 (HMGB1) in the patients with Acute Obstructive Suppurative Cholangitis-induced Sepsis

Abstract

Backgrounds: High Mobility Group Box 1 (HMGB1) is a proinflammatory cytokine which is found to play an active role during the pathogenesis of inflammatory processes. Our primary aim is to detect whether HMGB1 is involved in the pathogenesis of Acute Obstructive Suppurative Cholangitis (AOSC).

Methods: We collected samples of peripheral blood from 23 patients with AOSC and 23 healthy volunteers who served as normal controls. All were tested for the HMGB1 mRNA, the HMGB1 protein, Tumor Necrosis Factor - alpha (TNF - alpha) and Interleukin -10 (IL-10). HMGB1 mRNA was tested using RT-PCR. HMGB1 Protein was tested using Western blot. TNF-alpha and IL-10 were tested using ELISA.

Results: The expression of HMGB1 mRNA and HMGB1 Proteins were higher in AOSC group than that of normal controls ($P<0.01$) and it gradually decreased to normal level after the treatment of the disease ($P<0.01$). The content of TNF-alpha and IL-10 in peripheral blood of AOSC patients was significantly higher than the content of normal controls ($P<0.01$) and they decreased to normal level after the necessary treatment ($P<0.01$).

Conclusion: The level of HMGB1 mRNA and HMGB1 Protein are elevated in patients with AOSC which may have important role in the inflammation of bile duct and seems to be associated with the development of sepsis suggesting its importance in the management of AOSC-induced sepsis.

Keywords: Acute Obstructive Suppurative Cholangitis; HMGB1; Interleukin-10; TNF-alpha, Sepsis; Inflammation

Introduction

High mobility group box 1 (HMGB1) is chromatin binding protein which is normally located predominantly inside the nucleus of the cell. It is mainly responsible for transcription and growth factor. Recent studies of HMGB1 identified it as mediator of inflammation when it is released to the extracellular environment and has been demonstrated as a key factor for the development of pathogenesis of acute inflammation. In response to infection, HMGB1 acts as an important proinflammatory cytokine which may lead in the damage of cells and tissues. This process may continue to metabolic acidosis, multi-organ dysfunction syndrome (MODS) and sepsis. It is universally accepted that sepsis is a fatal which is mediated by inflammatory mediators like TNF-alpha, IL-10, HMGB1, etc. All these factors have important action in the startup, maintenance and resolution of inflammation. TNF-alpha and IL-10 are the early inflammatory factors which occur in the earlier phase during the onset of sepsis and even a minimal delay in the therapeutic method direct against the early inflammatory factors make the clinician difficult in the management of the disease. Serum HMGB1 was significantly elevated from 8 h to 72 h after the endotoxin exposure in comparison with early inflammatory mediators, TNF-alpha and Interleukin.[1,2] The treatment with the delayed administration of anti-HMGB1 antibodies[3], a box of HMGB1[3], and ethyl pyruvate beginning as late as the disappearance of plasma TNF-alpha and Interleukin significantly increases the survivals.[4] Thus, targeting HMGB1 for the

therapy of sepsis has wider role than targeting early inflammatory cytokines like TNF-alpha and IL-10 in the management of sepsis and recent studies has found many targeting agents against HMGB1 for the treatment of sepsis.[5,6]

Cholangitis is the inflammation of biliary ducts which usually begins extra-hepatic and easily spreads intra-hepatic, causing bacteremia. It was first described as clinical finding of fever, abdominal pain and jaundice by Charcot in 1977 known as "Charcot Triad" and later in 1959 by Reynolds with the addition of mental confusion and septic shock known as "Reynolds Pentad". Obstruction of biliary ducts and presence of superposing bacterial infection are common features in cholangitis. Obstruction of biliary tract is mostly caused by choledocholithiasis. Moreover, malignancy, benign strictures, and interventions to the biliary duct may also be the cause of biliary obstruction. Acute obstruction of biliary tract with the presence of pus is severe form of cholangitis know as Acute Obstructive Suppurative Cholangitis (AOSC) which has very high mortality if the drainage is delayed. It is experienced that the Charcot's triad and Reynold pentad are difficult to use in clinical practice for the management of cholangitis. Recently published "Updated Tokyo Guidelines for management of cholangitis and cholecystitis in 2013 is lack of evaluation and revision.[7] In our previous study, we found NF-kB which is very early mediator of inflammation is markedly increased in patients with severe acute cholangitis and degree of NF-kB is correlated with the degree of severity of the cholangitis.[8] Degree of NF-kB also correlates in the clinical

outcome. In another previous study with the patients of cholangitis, we observed that TREM-1, which is also an early inflammatory cytokine could significantly provide useful early indicator for sepsis.[9] But little to no research exits about the late inflammatory mediators in the patients with AOSC or sepsis induced by AOSC. Recent studies have reported associations between HMGB1 and inflammation.[10-12] Previously, we have found that HMGB1 can be potential valuable marker for the diagnosis of acute appendicitis.[13] But the correlation between HMGB1 and AOSC pathogenesis is not yet fully understood. Our objective of this current study was to determine HMGB1 with the inflammatory process of AOSC.

Materials and methods

All experimental protocols described in this study were approved by the research ethic review committee of the Second Affiliated Hospital of Chongqing Medical University and comply with the Chinese government guidelines. A total of 23 AOSC-induced sepsis patients admitted to the Department of Hepatobiliary Surgery, the Second Affiliated Hospital of Chongqing Medical University. P. R. China between November 2010 and June 2011 were randomly enrolled in prospective study. During that period, 23 healthy individuals were also randomly selected from the Physical Examination Center of the Second Affiliated Hospital, Chongqing Medical University, P. R. China with their written consent for involvement in this study for study as a control group. All the AOSC-induced sepsis patients and the control groups were between the ages of 18 years to 80 years old and ethnically similar (Han Chinese). The selection of AOSC-induced sepsis patients was based on Diagnostic criteria and severity assessment of acute cholangitis: Tokyo Guidelines[14] and International Sepsis Definitions Conference 2003.[15] Patients involved in this study were performed biliary drainage by via ERCP or PRC or Endoscopic Ultrasound-guided drainage or open surgical drainage.

The peripheral blood samples of each AOSC-induced sepsis patients were obtained pre-drainage, 12 hours post-drainage and 24 hours post-drainage, respectively.

Also, peripheral blood samples from control groups were obtained after overnight fasting.

Each sample was collected into 5 mL tubes, one with heparin and the other without heparin. The peripheral blood mononuclear cells (PBMCs) were immediately separated from the samples with heparin using a lymphocytes separating medium and a density gradient centrifuge. We wait until the blood clot for the tube without heparin. The serum was separated and taken out from the tube and then stored at -70 ^{0}C.

mRNA measurement by Real Time Polymerase Chain Reaction (RT-PCR)

Mononuclear cells were isolated from the blood sample using a lymphocytes separation medium (Bosoter, China), a ficoll density gradient isolating lymphocytes by centrifuging. The single step technique of acid guanidinium thiocyanate chloroform extraction was used for total RNA extraction according to the instructions provided by manufacture. We determined the concentration of purified total RNA by spectrophotometrically at the weave length of 280 nm. mRNA of HMGB1 in blood samples and β-actin were quantified in duplicate by Real Time - PCR. We removed the potential contaminated DNA with DNase I. We used 1 ug of total RNA from each sample for reverse transcription with an oligo (dT) and a Super Script II to generate first strand cDNA. Thermal cycling conditions were

done for 2 min at 94 ^{0}C followed by 35 cycles at 94 ^{0}C for 30 s and at 60 ^{0}C for 3 min on a Sequence Detection System (Applied Biosystems, Foster City, CS, USA). Each gene expression was normalized with β-actin mRNA content. Sequences of human primer for PCR were shown which follows: HMGB1 Bbox (127 bp) – 5'-GCG GAC AAG GCC CGT TA-3' (sense), 5'- AGA GGA AGA AGG CCG AAG GA – 3' (antisense); β-actin (255bp) – 5' – CAA AGA CCT GTA CGC CAA CA -3' (sense), 5' – GAA GCA TTT GCG GTG GAC – 3' (antisense).

HMGB1 Protein Measurement by Western Blotting Analysis

The difference between HMGB1 box gene expressions levels were analyzed using a Western Blotting Analysis. Proteins were quantified using Nanodrops 1000 (Thermo Scientific, Wilmington, DE, USA). Serum blood samples were denaturized at 100 0C for 5 min. Equal amount of proteins were loaded in each well for electrophoresis in 10% of SDS-polyacrylamide gels, then transferred to polyvinylidene fluoride micropoporous membranes (Millipore Corporation, Billerica, MA). The membrane containing the transferred proteins were blocked with 5% bovine serum albumin (BSA) and then incubated with rabit anti-HMGB1 polyclonal antibody (1:250; BD Pharmingen, San Diego CA) followed by an incubation with horseradish peroxidase-linked secondary antibodies (1:1000; Golden Bridge, Beijing, China). For the standardization and comparison, the membranes were also hybridized to a primary anti β-actin antibody (1:1000; Santa Cruz technology, santa Cruz, CA, USA) or antihistine H 3.1 antibody (1:1000;

SAB, Pearland, TX, USA). The bands appearing on film were analyzed with Gene Tools software (Syngene, Frederick, MD).

Enzyme-linked immunosorbent assay (ELISA)

The concentration of TNF-alpha and IL-10 in the blood serum was detected using commercial available ELISA kits (R and D Systems, Minneapolis, MN, USA) and performed according to the manufacturer's instructions.

These three statically analysis were completed with SPSS19.0 software for Windows (SPSS, Chicago, IL, USA). Data are presented as mean ±SEM unless otherwise indicated. A Kuskal-Wallis analysis was performed to ascertain that there is an overall difference, and Mann-Whitney U test was used to test difference between the groups. P values <0.05 were considered statically significant. P values <0.01 were considered notable statistical significant.

Results

Association of HMGB1 mRNA expression in Peripheral Blood Mononuclear Cells (PBMCs) of AOSC Patients

The total RNA of peripheral blood mononuclear cells (PBMCs) were extracted and the HMGB1 mRNA were amplified by RT-PCT to detect the expression of HMGB1 mRNA in PBMCs of AOSC patients (Figure 1). As shown in the Figure 3, the level of HMGB1 mRNA of PBMCs with AOSC patients was 0.82±0.04 which was significantly 62% higher than that of 0.31±0.06 ($P<0.01$) in healthy donors. We also found that the level of HMGB1 mRNA gradually decreased to 0.4±0.05 and 0.38±0.07 ($P<0.01$) after 12 hours and 24 hours post drainage respectively. The 12 hour post drainage level of HMGB1 mRNA was decreased to 32% compared with pre-drainage.

Association of HMGB1 protein in Peripheral Blood Mononuclear Cells (PBMCs) of AOSC Patients

The HMGB1 protein level of both the groups, the AOSC patients and the healthy donors, were tested using a Western Blotting Analysis from the prepared blood serum, which was stored at -70 ^{0}C (Figure 2). As in Figure 3, we found that the level of the HMGB1 protein in AOSC patients was 0.81±0.03, which was significantly 57% higher than that of 0.35±0.05 ($P<0.01$) in healthy donors. The level of HMGB1 proteins gradually decreased to 0.56±0.05 and 0.42±0.07 ($P<0.01$)

after 12 hours and 24 hours post-drainage. The 12 hour post drainage level of HMGB1 proteins decreased 37% compared to pre-drainage.

Association of TNF-alpha and IL-10 expression in Peripheral Blood Mononuclear Cells (PBMCs) of AOSC Patients

The level of TN- alpha and IL-10 proteins in the blood serum of each sample of AOSC patients and healthy donors was determined using commercial available ELISA kits. We found that the TNF-alpha and IL-10 were significantly higher than the contents in healthy donors and gradually decreased after biliary passage drainage (Figure 4). The average pre-drainage value of TNF-alpha was 469±35 pg/ml and it decreased to 243±26 pg/ml and 132±47 pg/ml after 12 hours and 24 hours post-drainage respectively, while it was 102±16 pg/ml in healthy controls. In the same way, the average pre-drainage value of IL-10 was 33.369±6.443 pg/ml and it decreased to 20.562±5.630 pg/ml and 13.742±3.374 pg/ml after 12 hours and 24 hours post-drainage respectively, while it was 11.490±1.088 pg/ml in healthy controls (Figure 5).

Discussion

Acute cholangitis is a systemic infection disease characterized by acute inflammation and biliary tract infection. Acute cholangitis occur due to the bacterial colonization within the biliary tract which increase the internal pressure of biliary tract and cause the backflow of bacteria and endotoxins within the circulatory system.[16,17] Acute Obstructive Suppurative Cholangitis (AOSC) is severe form of cholangitis where there is collection of pus in the biliary tract. Drainage of biliary tract should be performed as soon as possible in patients with AOSC otherwise translocation of bacteria into the bloodstream results in septicemia, an often fatal complication of acute cholangitis which induces severe organ damage and high mortality. There was no appropriate guideline for the diagnosis and management of acute cholangitis before TG13: Updated Tokyo Guidelines for the management of acute cholangitis and cholecystitis.[7] The symptoms and signs described as Charcot's triad and Reynold's pentad are difficult for the physicians to use in the diagnosis and management strategies for the clinical setting. The newly published TG13 is considered to have better diagnostic and management capacity. However, TG13 lacks enough feedback and revisions. Within one year of short time period, some studies found the diagnostic criteria of TG13 fails to express severe cases of Cholangitis that requires emergency biliary drainage.[18] Hence, management strategy of TG13 might not very specific for AOSC which is severe form of cholangitis in which emergency biliary drainage is

mandatory. Hence, there is need for further study in the diagnosis and management of AOSC. It will be a helpful step for the management of AOSC if there is marker that can be used to diagnose the AOSC-induced cholangitis. Recently, many studies have found that HMGB1 is an important agent for the diagnosis and management of sepsis. This study focus on the correlation of HMGB1 with the patients of AOSC.

These days many clinical studies, as well as experimental studies have described the release of HMGB1 during inflammation.[13,19] It is shown that HMGB1 is found as a mediator of inflammation when it is released extracellular. The extracellular release of HMGB1 might be either through active release which is by immune cells and passive secretion after cell is dead. Inside the nucleus, HMGB1 function as a protein for binding chromatin and help in the transcription process of gene.[20] Extracellular release of HMGB1 is due to translocation of HMGB1 from nucleus into the cytoplasm which involve process of acetylation[21], phosphorylation[22] and methylation.[23] Extracellular HMGB1 then start exerting biological expression via its receptors. HMGB1 signaling is mediated by several receptors which include Receptor for Advanced Glycation Endproduct (RAGE) and Toll-like receptors (TLRs).[24,25] During infection, endotoxins are produced by bacteria which activate monocytes and macrophages such as TNF-alpha, IL-10, etc. These cytokines promote HMGB1 translocation from nucleus into the cytoplasm and further goes into transcription process by themselves.[5,26,27] These produced HMGB1 acts

themselves as cytokines which further induces the stimulation of another inflammatory agents resulting in worsening of inflammation.

Our study demonstrated the expression of TNF-alpha, IL-10 and HMGB1 level were significantly high in the patients with AOSC than the normal control. After the drainage, the level of HMGB1, TNF-alpha and IL-10 with the patients with AOSC were decreased as infection was gradually decreased indicating that, with the exacerbation of AOSC, HMGB1 is positively correlated with the disease. Increased expression of TNF-alpha and IL-10 are also shown in non-inflammatory diseases whereas increased expression of HMGB1 is particularly seen in the peripheral blood mononuclear cells (PMCs) and serum with the patients with sepsis. Some recent studies have also reported that the severity of the disease increases with the increase in the expression of HMGB1. In severely burned patients, it is reported that the level of HMGB1 expression is significantly higher in dead patients in comparison to the survivors.[28] HMGB1 has been studies since more than four decades and in recent years it has gained its attention for the marker of sepsis. Our previous study has demonstrated that the HMGB1 expression can be novel marker for the diagnosis of acute appendicitis and also help the physician to decide the severity of the disease.[13] As TNF-alpha and IL-10 are early marker of inflammation whereas expression of HMGB1 is found up to late stage of the sepsis, its significance in sepsis patients can be higher than early inflammatory factors like TNF-alpha and IL-10. Currently, several animal and clinical studies have also

demonstrated protective effect of anti-HMGB1 agent on sepsis. It should be noted that if the sepsis is due to HMGB1, there will be an adequate time interval to detect it and give anti-HMGB1 for the therapeutic management of the sepsis.

In the patients with AOSC-induces sepsis, bacteria can easily enter the circulatory system as there is increased intra-ductal pressure due to blockage of biliary tract. These bacteria produces endotoxins and proinflammatory agents. The produced proinflammatory agents activate monocytes and macrophages which cause the HMGB1 to release in the extracellular environment. The released HMGB1 act itself as proinflammatory factor which worsen the inflammation as well as affect the distal organs.[26,27] From this point of view, the increased level of HMGB1 could directly correlated with the severity of ASOC-induced sepsis and hence can be useful in the diagnostic marker with the patients of AOSC. Also, controlling the expression of HMGB1 by anti-HMGB1 agent might improve the efficacy in the therapy of AOSC by managing systemic inflammation and reducing organ injury by sepsis. However, there is lack of clinical evidence in suppressing increased expression of HMGB1 in sepsis patients and this need to clarify by the results of clinical trials.

Conclusion

Our study has demonstrated increased expression of HMGB1 in serum and peripheral blood mononuclear cells of AOSC-induced sepsis patients which may play important role for the progression of sepsis with the patients with AOSC. The sample size of our study is small and we have not included the inflammation due to other disorders which are the important parameters for affecting sensitivity and specificity of HMBG1 in the patients of AOSC. The mechanism of HMGB1 with the patients of AOSC remain unclear and some work remain to be done. This result is not designed to control AOSC-induced sepsis through anti-HMGB1 therapy and this needs to be further researched. While we have yet to complete further researches to demonstrate the sensitivity and specificity of HMGB1 in the diagnosis and therapy for the patients with AOSC, combined with other diagnostic markers, HMGB1 could provide a useful biomarker for AOSC-induced sepsis.

Disclosure

The authors report no conflict of interest related to this work.

References

1. Wang H, Bloom O, Zhang M, et al. HMG-1 as a late mediator of endotoxin lethality in mice. *Science.* Jul 9 1999;285(5425):248-251.
2. Yang H, Ochani M, Li J, et al. Reversing established sepsis with antagonists of endogenous high-mobility group box 1. *Proceedings of the National Academy of*

Sciences of the United States of America. Jan 6 2004;101(1):296-301.

3. Li X, Wang LK, Wang LW, Han XQ, Yang F, Gong ZJ. Blockade of high-mobility group box-1 ameliorates acute on chronic liver failure in rats. *Inflammation research : official journal of the European Histamine Research Society ... [et al.].* Jul 2013;62(7):703-709.
4. Ulloa L, Ochani M, Yang H, et al. Ethyl pyruvate prevents lethality in mice with established lethal sepsis and systemic inflammation. *Proceedings of the National Academy of Sciences of the United States of America.* Sep 17 2002;99(19):12351-12356.
5. Fink MP. Neuropeptide modulators of high mobility group box 1 secretion as potential therapeutic agents for severe sepsis. *The American journal of pathology.* May 2008;172(5):1171-1173.
6. Yoo H, Ku SK, Baek YD, Bae JS. Anti-inflammatory effects of rutin on HMGB1-induced inflammatory responses in vitro and in vivo. *Inflammation research : official journal of the European Histamine Research Society ... [et al.].* Mar 2014;63(3):197-206.
7. Takada T, Strasberg SM, Solomkin JS, et al. TG13: Updated Tokyo Guidelines for the management of acute cholangitis and cholecystitis. *Journal of hepato-biliary-pancreatic sciences.* Jan 2013;20(1):1-7.
8. Gong JP, Liu CA, Wu CX, Li SW, Shi YJ, Li XH. Nuclear factor kB activity in patients with acute severe cholangitis. *World journal of gastroenterology : WJG.* Apr 2002;8(2):346-349.
9. Liao R, Liu Z, Wei S, Xu F, Chen Z, Gong J. Triggering receptor in myeloid cells (TREM-1) specific expression in peripheral blood mononuclear cells of sepsis patients with acute cholangitis. *Inflammation.* Jun 2009;32(3):182-190.
10. El Gazzar M. HMGB1 modulates inflammatory responses in LPS-activated macrophages. *Inflammation research : official journal of the European Histamine Research Society ... [et al.].* Apr 2007;56(4):162-167.
11. Kao RL, Xu X, Xenocostas A, et al. Induction of acute lung inflammation in mice with hemorrhagic shock and resuscitation: role of HMGB1. *Journal of inflammation.* 2014;11(1):30.
12. Hou C, Kong J, Liang Y, et al. HMGB1 contributes to allergen-induced airway remodeling in a murine model of chronic asthma by modulating airway inflammation and activating lung fibroblasts. *Cellular & molecular immunology.* Aug 25 2014.
13. Wu C, Sun H, Wang H, et al. Evaluation of high mobility group box 1 protein as a presurgical diagnostic marker reflecting the severity of acute appendicitis. *Scandinavian journal of trauma, resuscitation and emergency medicine.* 2012;20:61.
14. Wada K, Takada T, Kawarada Y, et al. Diagnostic criteria and severity assessment of acute cholangitis: Tokyo Guidelines. *Journal of hepato-biliary-pancreatic surgery.* 2007;14(1):52-58.
15. Levy MM, Fink MP, Marshall JC, et al. 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference. *Critical care medicine.* Apr

2003;31(4):1250-1256.

16. Kimura Y, Takada T, Strasberg SM, et al. TG13 current terminology, etiology, and epidemiology of acute cholangitis and cholecystitis. *Journal of hepato-biliary-pancreatic sciences.* Jan 2013;20(1):8-23.
17. Mosler P. Diagnosis and management of acute cholangitis. *Current gastroenterology reports.* Apr 2011;13(2):166-172.
18. Shinya S, Sasaki T, Yamashita Y, et al. Procalcitonin as a useful biomarker for determining the need to perform emergency biliary drainage in cases of acute cholangitis. *Journal of hepato-biliary-pancreatic sciences.* Oct 2014;21(10):777-785.
19. Wu CX, Sun H, Liu Q, Guo H, Gong JP. LPS induces HMGB1 relocation and release by activating the NF-kappaB-CBP signal transduction pathway in the murine macrophage-like cell line RAW264.7. *The Journal of surgical research.* Jun 1 2012;175(1):88-100.
20. Stott K, Tang GS, Lee KB, Thomas JO. Structure of a complex of tandem HMG boxes and DNA. *Journal of molecular biology.* Jun 30 2006;360(1):90-104.
21. Li Y, Gan CP, Zhang S, et al. FIP200 is involved in murine pseudomonas infection by regulating HMGB1 intracellular translocation. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology.* 2014;33(6):1733-1744.
22. Ge X, Antoine DJ, Lu Y, et al. High mobility group box-1 (HMGB1) participates in the pathogenesis of alcoholic liver disease (ALD). *The Journal of biological chemistry.* Aug 15 2014;289(33):22672-22691.
23. Wu F, Zhao ZH, Ding ST, Wu HH, Lu JJ. High mobility group box 1 protein is methylated and transported to cytoplasm in clear cell renal cell carcinoma. *Asian Pacific journal of cancer prevention : APJCP.* 2013;14(10):5789-5795.
24. Park JS, Gamboni-Robertson F, He Q, et al. High mobility group box 1 protein interacts with multiple Toll-like receptors. *American journal of physiology. Cell physiology.* Mar 2006;290(3):C917-924.
25. Ivanov S, Dragoi AM, Wang X, et al. A novel role for HMGB1 in TLR9-mediated inflammatory responses to CpG-DNA. *Blood.* Sep 15 2007;110(6):1970-1981.
26. Pisetsky DS. The expression of HMGB1 on microparticles released during cell activation and cell death in vitro and in vivo. *Molecular medicine.* 2014;20:158-163.
27. Rendon-Mitchell B, Ochani M, Li J, et al. IFN-gamma induces high mobility group box 1 protein release partly through a TNF-dependent mechanism. *Journal of immunology.* Apr 1 2003;170(7):3890-3897.
28. Huang LF, Yao YM, Dong N, Yu Y, He LX, Sheng ZY. Association of high mobility group box-1 protein levels with sepsis and outcome of severely burned patients. *Cytokine.* Jan 2011;53(1):29-34.

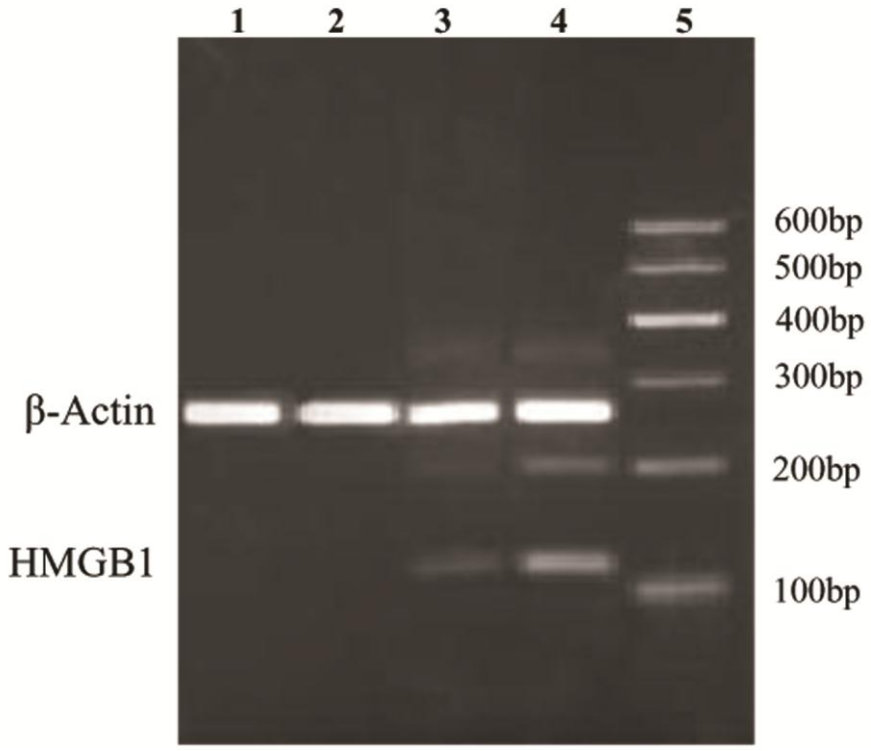

Figure 1 Expression of high-mobility group box 1 mRNAin peripheral blood mononuclear cells by real-time polymerase chain reaction analysis.

Notes: Total RNAwas obtained from peripheral blood mononuclear cells of the control group and from acute obstructive suppurative cholangitis-induced sepsis patients (before the drainage, 12 hours after the drainage, and 24 hours after the drainage, respectively). Electrophoresis of high-mobility group box 1 gene fragment amplification product is shown. Lane 1 represents polymerase chain reaction product of control group, lane 2 represents polymerase chain reaction product of AOSC-induced sepsis group 24 hours after the drainage, lane 3 represents polymerase chain reaction product of AOSC-induced sepsis group 12 hours after the drainage, lane 4 represents polymerase chain reaction product of AOSC-induced sepsis group before the drainage and lane 5 represents DL2000 marker.

Abbreviations: AOSC, acute obstructive suppurative cholangitis; HMGB1, high-mobility group box 1.

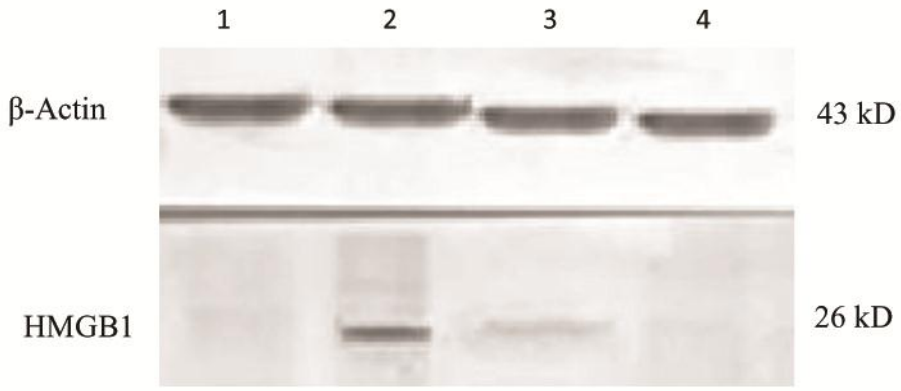

Figure 2 Western blotting analysis of high-mobility group box 1 protein expression in peripheral blood mononuclear cells.
Notes: Western blotting was performed to detect protein level of high-mobility group box 1. Total protein was extracted from peripheral blood mononuclear cells. Lane 1 represents the control, lane 2 represents preoperation, lane 3 represents postoperation 12 hours, and lane 4 represents postoperation 24 hours.
Abbreviation: HMGB1, high-mobility group box 1.

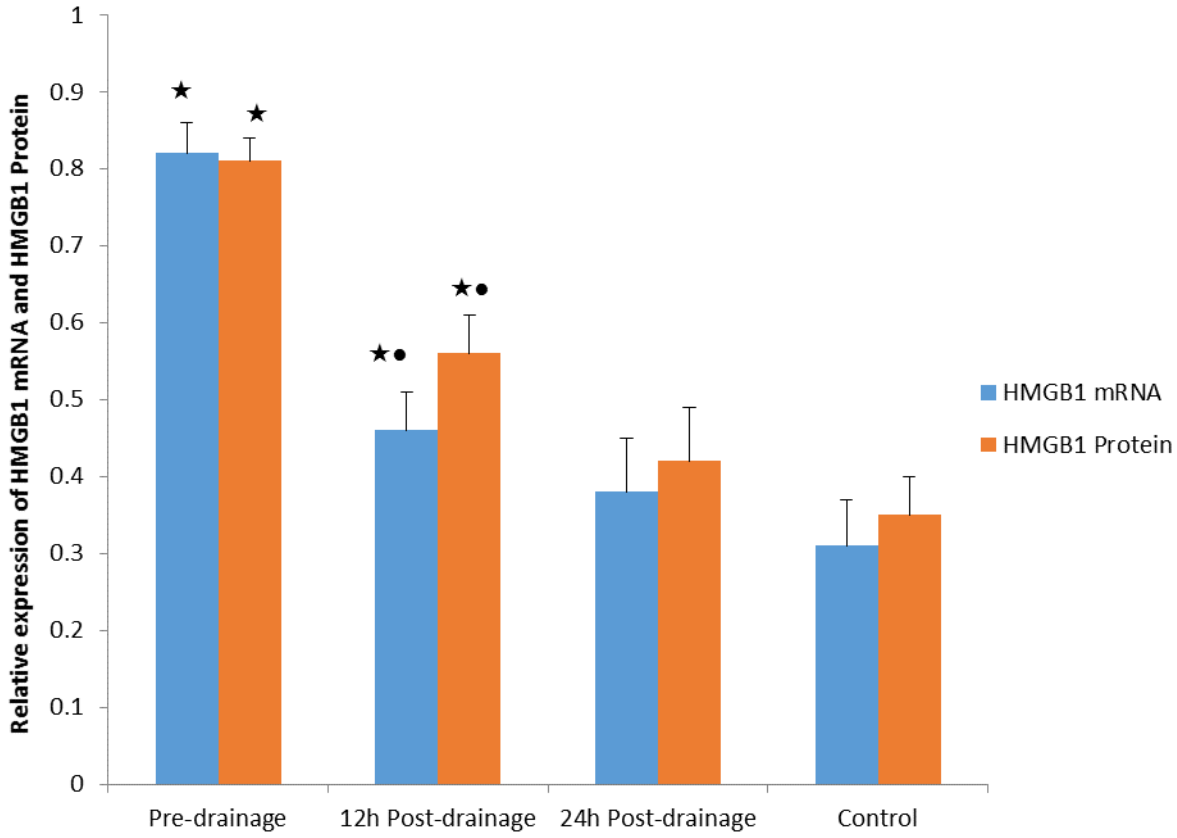

Figure 3 Comparison of high-mobility group box 1 (HMGB1) mRNAand high-mobility group box 1 protein expression among patients with acute obstructive suppurative cholangitis and controls (n=23).

Notes: Compared with control, P,0.01; •compared with predrainage, P,0.01.

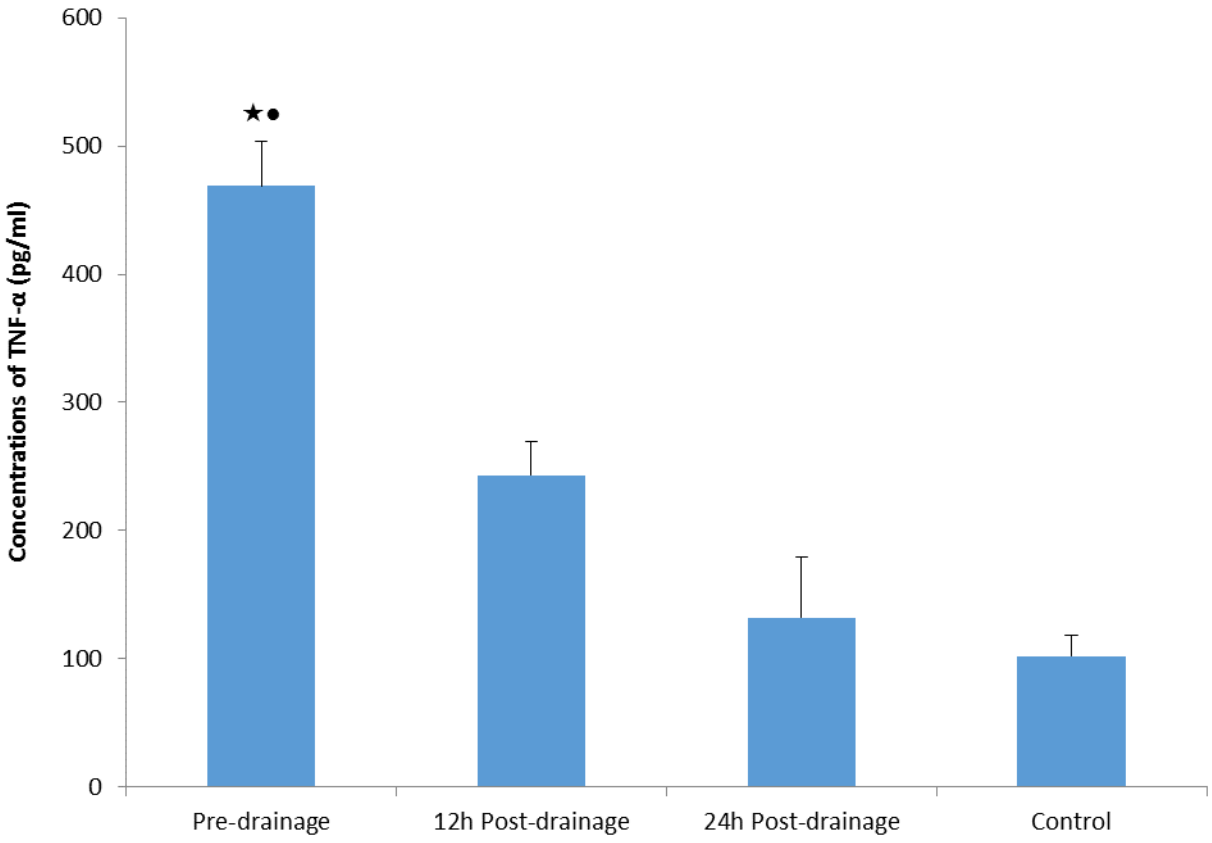

Figure 4 The analysis of tumor necrosis factor alpha by enzyme-linked immunosorbent assay (ELISA) in serum of operated and healthy controls.
Notes: Tumor necrosis factor alpha (TNF-α) was quantitated using an ELISAkit. Data are expressed as median for each sample. Experiments were performed at least three times, getting similar results. Compared with control, P,0.01; •compared with 12 hours postdrainage, P,0.01.

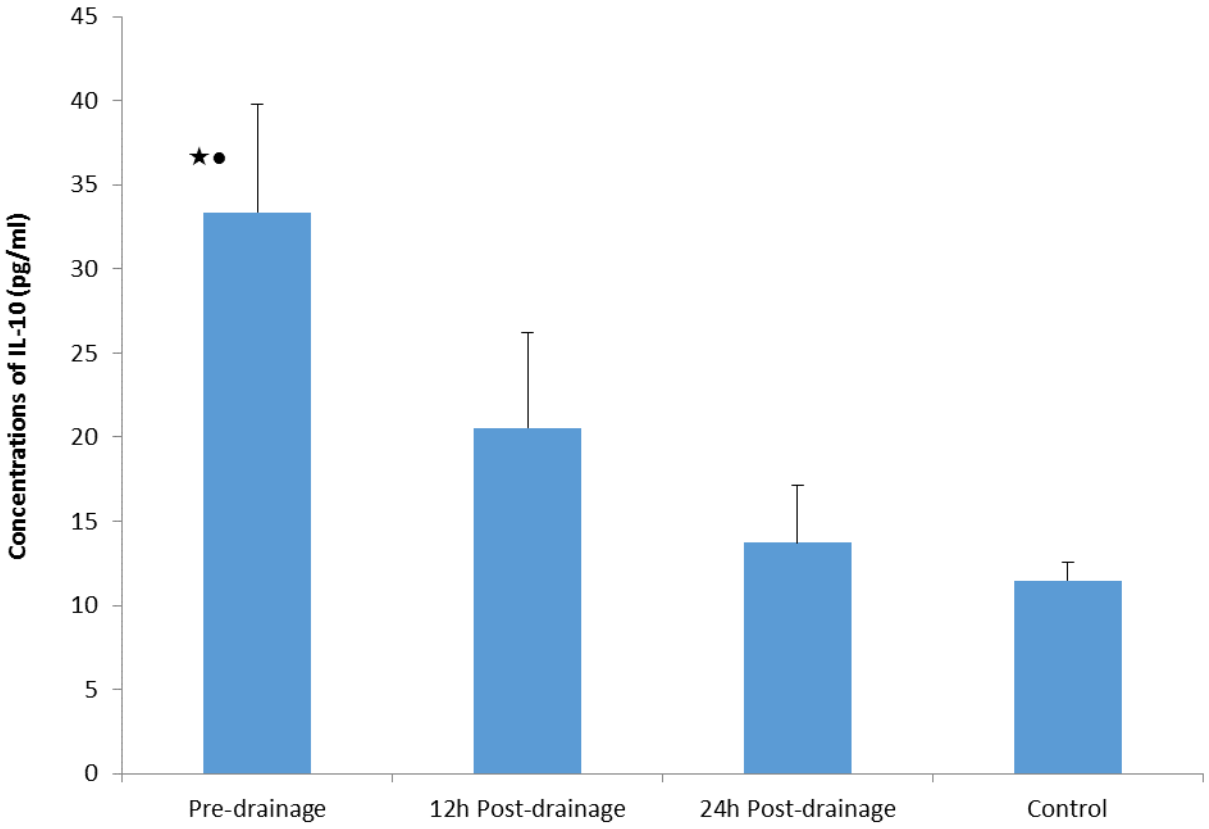

Figure 5 The analysis of interleukin 10 (IL-10) by enzyme-linked immunosorbent assay (ELISA) in serum of operated and healthy controls.

Notes: Tumor necrosis factor alpha was quantitated using ELISAkit. Data are expressed as median for each sample. Experiments were performed at least three times getting similar results. Compared with control, P,0.01; •compared with 12 hours postdrainage, P,0.01.

Table 1. Clinical Characteristic of Patients with AOSC and Controls

		AOSC
Healthy Control (n=23)		
Age	52.5±21.5	51.5±20.70
Sex (M/F)	13/10	12/11
Temperature (℃)	39.6±.95	36.6±.46
White Blood Cell (cells/μl)	18.27±3.59	6.75±1.67
Neutrophil (cells/μl)	16.52±3.11	3.69±0.41
Monocyte (cells/μl)	0.90±0.17	0.21±0.06
Liver Function		
AST (U/L)	207.51±40.67	27.22±9.72
ALT(U/L)	186.83±43.31	22.13±6.47
DB(μmol/L)	101.06±22.74	7.48±4.74
IB(μmol/L)	75.97±17.54	5.56±2.28
TBA(μmol/L)	167.48±36.31	9.48±2.43

AOSC = Acute Obstructive Suppurative Cholangitis, AST = Aspartate Aminotransferase, ALT = Alanine Aminotransferase, DB = direct bilirubin, IB = Indirect Bilirubin, TBA = Total Bile Acid

(II) Expression of Scavenger Receptor A in Rat's Liver Tissue during Acute Obstructive Cholangitis and Its Significance

Abstract:

Objective: Acute Obstructive Cholangitis (AOC) is a common disease which leads to sepsis, shock and multiple organ dysfunction syndrome (MODS). Liver is the largest and the major organ for the defense mechanisms during the sepsis. Our aim was to investigate the expression of Scavenger Receptor A (SRA) in rat's liver tissue during AOC and its relation with the inflammatory mediators and hepatic injury caused by endotoxins.

Methods: Mouse model of AOC were reproduced by ligating choledochus and injecting *Escherichia coli* O_{111}B4 into it. The concentration of plasma endotoxins were assayed by litmus test. The level of tumor necrosis factor-alpha (TNF-alpha) and Interleukin-6 (IL-6) in plasma were determined with enzyme-linked immunosorbent assay (ELISA). The expression of SRA protein in liver tissue was assayed by immunohistochemistry, and the expression of SRA mRNA in liver tissue were detected via light microscope.

Results: The plasma endotoxins concentrations in AOC were progressively increased with the time prolonged. With increasing endotoxin, the level of TNF-alpha and IL-6 were also markedly increased. The SRA protein and mRNA expression were obviously decreased with prolonged experimental time. There were significant difference among experimental groups and control groups

(P<0.01). Liver histopathological study showed that the hepatic injury was gradually aggravated with the time prolonged. Massive inflammatory cells infiltration and large-area hepatocytes degeneration and necrosis were found in later period.

Conclusion: The expression of SRA in Kupffer Cells (KCs) in liver tissue gradually decreases with progressive increasing plasma endotoxins level in AOC. The decreased expression of SRA is responsible for endotoxin hepatic injury. It might be one of the important mechanism for the conversion of KCs from immune defensive cells to inflammatory response cells.

Key Words: Acute Obstructive Cholangitis; Scavenger Receptor A; Endotoxemia; Cytokine, Endotoxin Hepatic Injury

Introduction

Liver is vital organ which has many functions including immunological tolerance for foreign antigens. Liver diseases are major health problems with significant mortality.(Guan and He 2013) Acute obstructive cholangitis (AOC) commonly leads to sepsis, septic shock and multiple organ dysfunction syndrome (MODS).(Takada et al. 2013) Liver is the largest organ for defense in sepsis.(Hilliard et al. 2015) Kupffer cells play vital role in defense by clearing bacterium and endotoxin. (Bilzer et al. 2006; Hutchins et al. 2013) They also have important role in the pathogenesis of hepatic injury in sepsis and MODS.(Sato et al. 2014; Kim et al. 2011; Rivera et al. 2007) Although pathological and immunological mechanism of hepatic injury due to inflammatory diseases have not been completely understood, activation of hepatic endotoxin is critical event in inflammatory process.(Heymann et al. 2015) Scavenger Receptor A (SRA) is a transmembrane glycoprotein and mainly distributed in hepatic Kupffer cells.(Prabhudas et al. 2014) The SRA reflects defense reaction that mediates macrophage clearance and inactivation of endotoxin.(Kelley et al. 2014)

We aim establish animal model of AOC in Wistar rats to investigate the expression of SRA and its relations to endotoxin, tumor necrosis factor (TNF-α), interleukin (IL-6) and hepatic injury. The histological changes in Kupffer cells will provide evidence for its changing role from immune defense to inflammatory response cells during AOC. These observations may provide basis of therapeutic regimen targeted to protect liver cell damage during AOC, sepsis and MODS.

Materials and Methods

Animal

All experimental protocols described in this study were approved by the research ethics review committee of the Second Affiliated Hospital of Chongqing Medical University and comply with the Chinese government guidelines. Ninety Wistar rats of both male and female weighing 200±20g were used. The animals were divided randomly into three groups. The first group (n=30) was the acute cholangitis group (AC). We set up AOC animal model by ligating choledochus and injecting Escherichia coli $O_{111}B4$ (5×10^9 cfu/ml) 0.2ml into it. The second group (n=30) was the bile duct ligation group (BDL), whose choledochous ducts were ligated and injected into 0.2ml 0.9% normal sodium. The third group (n=30) was the sham operation group (SO). The rats were sacrificed immediately at 0, 3, 6, 12 and 24 hours, respectively. There were 6 rats at each time point. Plasma and liver tissue were extracted and stored at -20℃ and -70℃ for later use.

Methods

Endotoxin concentration in plasma

The Limulus Amebocyte Lysate assay kits were bought from Shanghai Yihua Medical Technology Inc. The concentrations of endotoxin in plasma were assayed by limulus test according to the manufacturer's instructions. The presence of the endotoxin was detected at 545 nm using the Bio-tek Kcjunior microplate reader.

TNF-α and IL-6 levels in plasma

The levels of TNF-α and IL-6 in plasma were measured with ELISA kits (Boster Inc, Wuhan) designed for the determination of rat cytokines following manufacturer's instructions. The cytokines were detected at 450 nm still using the Bio-tek Kcjunior microplate reader.

Expression of SR-A Protein

The expression of SR-A protein in liver tissue was assayed by standard immunohistochemistry examination. The first antibody was goat anti-mouse SRA monoclonal antibody, which was bought from Santa Cruz Inc. The second antibody got from Wuhan Boster Inc, which was a rabbit anti-goat antibody. Placing the sections under 400×light microscope, we might figure out average Immunohistochemistry (IHC) (+) Masculine Cell Population (MCP) by randomly selecting five visual fields to count IHC (+) MCP.

Expression of SRA mRNA

The expression of SRA mRNA in liver tissue was measured by real time polymerase chain reaction (RT-PCR). The primer of SRA mRNA and the primer of β-actin mRNA as control were designed according to method of Singh A et al.(Singh et al. 2015) The images, got from gel electrophoresis of RT-PCR amplification products, could stand for relative expression of SRA mRNA by being automatic analyzed in BioRad Image Pro-Plus system. That was to say, the relative expression of SRA mRNA was equal to $IOD_{SR\text{-}A}/IOD_{\beta\text{-}actin}$.

Histopathological study in liver tissue

The liver tissue samples were fixed in 10% neutral- buffered formalin. After 48 hours of fixation, liver tissues were embedded in paraffin. 4 micrometer sections were sectioned, and stained with hematoxylin and eosin for conventional histopathological evaluation.

Statistical analysis

Results are expressed as mean ± SEM. Statistical analysis were performed using software of SPSS 20.0. The difference among each group were analyzed by t test. A *P* value below 0.05 was considered to be significant.

Results

The changes of plasma endotoxin levels

The plasma endotoxin concentrations in AOC group had increased obviously at postoperative 3 hours, and progressively risen with prolonged experimental time. It reached peak at 24 hours. Compared with BDL group and SO group, there were significant differences among three groups ($P<0.01$). The levels of endotoxin in BDL group at 24h slightly increased, while those in SO group have no obvious varieties in various time points (**Figure 1-1**).

The changes of plasma TNF-α and IL-6 levels

The plasma TNF-α content in AOC group had soar evidently after postoperative 3 hours, while those of IL-6 just started increase at 3 hours and increased strikingly at 6 hours. Both of them continued to rise with prolonged experimental time and reached peak at 24 hours. Compared with the BDL group and the SO group, there were marked differences among three groups ($P<0.01$). The levels of TNF-α in BDL group mildly increased at 12h and 24h, while those of IL-6 in BDL group just gently increased at 24h. The levels of TNF-α and IL-6 in SO group in various time points have no evident varieties (**Figure 2-1, and Figure 2-2**).

The expression of SRA protein in liver tissue

We have found significant difference in the expression of SRA protein in liver tissue in AOC group compared with BDL group and SO group ($P<0.01$) (**Figure 3-1**). Immunohistochemistry showed that the brown positive products of SRA expression were mainly located on the surface of Kupffer cells and were diffusely

distributed inside liver tissue in AOC group at 0h (**Figure 3-2)**. The expression of SRA in AOC group had decreased at 3 hours and gradually descended with prolonged experimental time. It was very obvious at 24 hours (**Figure 3-3 ~ Figure 3-6**).

The expression of SRA mRNA in liver tissue

RT-PCR showed that the expression of SRA mRNA in AOC group had descended at 3 hours and progressively decreased with prolonged experimental time. At 24 hours, compared with the BDL group and SO group, AOC group have very significant differences ($P<0.01$). The expression of SRA mRNA in BDL and SO groups have not evident changes. We demonstrated furthermore the fact from gene that SRA expression in mRNA was in accordance with that in protein. The expression of SRA in gene and protein gradually decreased with progressive increasing plasma endotoxin levels. (**Figure 4-1 ~ Figure 4-2**).

Histopathological study in liver tissue

Histopathological study via light microscope have found that the hepatic injury gradually aggravated with prolonged experimental time in AOC group. Many inflammatory infiltration mainly consisting of neutrophil and expansion of central veins were found at 3h (**Figure 5-1**), focal inflammation were found in liver parenchyma at 6h (**Figure 5-2**), obvious expansion of portal area, massive mixing inflammatory cells infiltration were found at 12h (**Figure 5-3**), large-area hepatocyte degeneration and necrosis were found at 24h (**Figure 5-4**). In BDL group, manipulus inflammatory cells infiltration were found in portal area, no

obvious inflammatory cells were found in liver parenchyma (**Figure 5-5**). No obvious histopathological changes are found in SO group liver tissue (**Figure 5-6**).

Discussion

Acute obstructive cholangitis (AOC) is a type of cholangitis with the symptom of right upper abdominal pain, jaundice, chills with fever. In severe cases of AOC, sign of central nervous system such as lethargy, disorientation, or coma, combined with septic shock are involved.(Liao et al. 2009; Gong et al. 2002) It easily lead to systematic inflammatory response syndrome (SIRS) and MODS. Mortality rate of AOC is high if delayed in treatment which make the high mortality in nonmalignant diseases of biliary tract. Intervention for drainage of obstructed biliary tract is the therapeutic principle. However, there is still a high mortality even after the drainage as it already leads to sepsis and MODS in early stage of this disease. So, postoperative management is still very vital.

Liver is the largest and most important defense organ in sepsis. Kupffer cells are resident macrophages which reside within the lumen of liver sinusoids. They constitute 80-90 percent of the tissue macrophages present in the body(Bilzer et al. 2006). The surface of Kupffer cell has different LPS receptors such as CD14, TLRs, CD11a/CD1lb/CD18, CD1lc/CD18, SRA, etc. Kupffer cells get contact with LPS of portal vein receiving from gut where they play important role in defense by clearing bacterium and endotoxin. On the other hand, they also serve as mediators of inflammation, especially on endotoxin hepatic injury via releasing various cytokines (IL-6, TNF- α, etc.).(Sato et al. 2014) At present it is reported that Kupffer cells not only play important role in occurrence and development of liver diseases but also in liver’s ischemia-reperfusion injury.(Suyavaran et al. 2015)

SRA, which is first described by Brown and Goldstein in 1970s is a superfamily of membrane-bond receptors that were initially thought to bind and internalized modified low-density lipoprotein (LDL), though it is currently known to bind to variety of ligands including endogenous proteins and pathogens.(Goldstein et al. 1979) Currently, SRA is classified into 10 eukaryote families, defined as Classes A-J.(Zani et al. 2015) SRA is mainly distributed in various kinds of tissue macrophages, particularly in hepatic kupffer cells, spleen and lymph node macrophages. It is an important defensive receptor in macrophage surface, which can combine with bacterial lipopolysachharide (LPS, the important element of endotoxin). It plays important roles in defensive reaction which mediates macrophages clearing and inactivating endotoxin.

After establishment of AOC animal model, we assayed plasma endotoxin level in various time points. We found the concentration of plasma endotoxin increased with prolonged time which indicates the success of establishment of rat model.

Immunohistochemistry staining shows the expression of SRA gradually decreases with experimental time which means, it gradually decreases with increasing LPS. In the meantime, the expression of SRA mRNA also progressively decreases with the increase of LPS. Furthermore, we demonstrated SRA expression in mRNA was in accordance with that in protein. The expression of SRA in mRNA and Protein gradually decreases with progressive increasing plasma endotoxin levels. The expression of SRA mononuclear macrophages during endotoxemia has been widely reported.(Ozment et al. 2012; Zhao et al. 2015) One recent study suggest that

SRA is needed for LPS induced inflammatory responses in macrophages.(Yu et al. 2012) SRA is now appreciated to be multifunctional. Gradually, it is realized that SRA has contribution in defense mechanism.(Zuo et al. 2013)

Sepsis or MODS remains one of the major causes of death in AOC.(Huggett et al. 2014) Binding of LPS to macrophages could induce the release of cytokine and inflammatory mediators, leading to organ damage.(Singh et al. 2015) In our study, the plasma level of cytokines, TNF-alpha and IL-6 in AOC group significantly increased at 6h after operation and progressively increased with increasing level of plasma endotoxin. These findings indicate that inflammatory response is progressively enhanced in the liver tissue in AOC, which had positive relation with plasma endotoxin.

In this study, correlation analysis showed that changes in the levels of TNF-alpha and IL-6 in plasma were negatively correlated with the expression of SRA mRNA and protein. Meanwhile, in one previous study, blocking expression of SRA on kupffer cells could promote cytokine production. It is already reported that TNF-alpha and IL-6 has been related with endotoxin mediating inflammation and liver injury.(Zhang et al. 2015; Zhou et al. 2015) The reduced SRA expression leads to decrease on clearing and inactivating endotoxin, while endotoxin activates more Kupffer cells.(Xie et al. 2001; Jiang et al. 2003) Therefore, with participation of CD14 (another important receptor in surface of Kupffer cells, which is concerned with activation of Kupffer cells), Kupffer cells turn into effector cells. The release of TNF- α and IL-6 are increased, which result in more down-regulation of SRA

expression and up-regulation of CD14 expression.

The LPS-induced hepatic injury gradually aggravated with prolonged experimental time in AOC group. On Light microscope, the liver tissue showed different injuries characterized by infiltration of inflammatory cells, hepatocyte denaturation and necrosis. The degree of hepatic injury were related to endotoxin and correlated negatively with SRA. Beside the surgical interventions, we can add one new method in the management of AOC by controlling the function of Kupffer cells, especially controlling the expression of SRA.

Conclusion

In rat AOC model the expression of SRA from liver Kupffer cells decreased gradually with increasing plasma endotoxin, TNF-α and IL-6 leading to progressive liver injury of hepatocyte necrosis.

References

Bilzer M, Roggel F, Gerbes AL (2006) Role of Kupffer cells in host defense and liver disease. Liver Int 26 (10):1175-1186. doi:10.1111/j.1478-3231.2006.01342.x

Goldstein JL, Ho YK, Basu SK, Brown MS (1979) Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. Proceedings of the National Academy of Sciences of the United States of America 76 (1):333-337

Gong JP, Liu CA, Wu CX, Li SW, Shi YJ, Li XH (2002) Nuclear factor kB activity in patients with acute severe cholangitis. World J Gastroenterol 8 (2):346-349

Guan YS, He Q (2013) A current update on the rule of alternative and complementary medicine in the treatment of liver diseases. Evid Based Complement Alternat Med 2013:321234. doi:10.1155/2013/321234

Heymann F, Peusquens J, Ludwig-Portugall I, Kohlhepp M, Ergen C, Niemietz P, Martin C, van Rooijen N, Ochando JC, Randolph GJ, Luedde T, Ginhoux F, Kurts C, Trautwein C, Tacke F (2015) Liver inflammation abrogates

immunological tolerance induced by Kupffer cells. Hepatology 62 (1):279-291. doi:10.1002/hep.27793

Hilliard KL, Allen E, Traber KE, Kim Y, Wasserman GA, Jones MR, Mizgerd JP, Quinton LJ (2015) Activation of Hepatic STAT3 Maintains Pulmonary Defense during Endotoxemia. Infect Immun. doi:10.1128/IAI.00464-15

Huggett MT, Culver EL, Kumar M, Hurst JM, Rodriguez-Justo M, Chapman MH, Johnson GJ, Pereira SP, Chapman RW, Webster GJ, Barnes E (2014) Type 1 autoimmune pancreatitis and IgG4-related sclerosing cholangitis is associated with extrapancreatic organ failure, malignancy, and mortality in a prospective UK cohort. Am J Gastroenterol 109 (10):1675-1683. doi:10.1038/ajg.2014.223

Hutchins NA, Chung CS, Borgerding JN, Ayala CA, Ayala A (2013) Kupffer cells protect liver sinusoidal endothelial cells from Fas-dependent apoptosis in sepsis by down-regulating gp130. Am J Pathol 182 (3):742-754. doi:10.1016/j.ajpath.2012.11.023

Jiang JX, Chen YH, Xie GQ, Ji SH, Liu DW, Qiu J, Zhu PF, Wang ZG (2003) Intrapulmonary expression of scavenger receptor and CD14 and their relation to local inflammatory responses to endotoxemia in mice. World J Surg 27 (1):1-9. doi:10.1007/s00268-002-6548-5

Kelley JL, Ozment TR, Li C, Schweitzer JB, Williams DL (2014) Scavenger receptor-A (CD204): a two-edged sword in health and disease. Crit Rev Immunol 34 (3):241-261

Kim TH, Lee SH, Lee SM (2011) Role of Kupffer cells in pathogenesis of sepsis-induced drug metabolizing dysfunction. FEBS J 278 (13):2307-2317. doi:10.1111/j.1742-4658.2011.08148.x

Liao R, Liu Z, Wei S, Xu F, Chen Z, Gong J (2009) Triggering receptor in myeloid cells (TREM-1) specific expression in peripheral blood mononuclear cells of sepsis patients with acute cholangitis. Inflammation 32 (3):182-190. doi:10.1007/s10753-009-9119-1

Ozment TR, Ha T, Breuel KF, Ford TR, Ferguson DA, Kalbfleisch J, Schweitzer JB, Kelley JL, Li C, Williams DL (2012) Scavenger receptor class a plays a central role in mediating mortality and the development of the pro-inflammatory phenotype in polymicrobial sepsis. PLoS Pathog 8 (10):e1002967. doi:10.1371/journal.ppat.1002967

Prabhudas M, Bowdish D, Drickamer K, Febbraio M, Herz J, Kobzik L, Krieger M, Loike J, Means TK, Moestrup SK, Post S, Sawamura T, Silverstein S, Wang XY, El Khoury J (2014) Standardizing scavenger receptor nomenclature. J Immunol 192 (5):1997-2006. doi:10.4049/jimmunol.1490003

Rivera CA, Adegboyega P, van Rooijen N, Tagalicud A, Allman M, Wallace M (2007) Toll-like receptor-4 signaling and Kupffer cells play pivotal roles in the pathogenesis of non-alcoholic steatohepatitis. J Hepatol 47 (4):571-579. doi:10.1016/j.jhep.2007.04.019

Sato A, Nakashima H, Nakashima M, Ikarashi M, Nishiyama K, Kinoshita M, Seki S (2014) Involvement of the TNF and FasL produced by CD11b Kupffer

cells/macrophages in CCl4-induced acute hepatic injury. PLoS One 9 (3):e92515. doi:10.1371/journal.pone.0092515

Singh A, Feng Y, Mahato N, Li J, Wu C, Gong J (2015) Role of high-mobility group box 1 in patients with acute obstructive suppurative cholangitis-induced sepsis. J Inflamm Res 8:71-77. doi:10.2147/JIR.S77539

Suyavaran A, Ramamurthy C, Mareeswaran R, Subastri A, Lokeswara Rao P, Thirunavukkarasu C (2015) TNF-alpha suppression by glutathione preconditioning attenuates hepatic ischemia reperfusion injury in young and aged rats. Inflamm Res 64 (1):71-81. doi:10.1007/s00011-014-0785-6

Takada T, Strasberg SM, Solomkin JS, Pitt HA, Gomi H, Yoshida M, Mayumi T, Miura F, Gouma DJ, Garden OJ, Buchler MW, Kiriyama S, Yokoe M, Kimura Y, Tsuyuguchi T, Itoi T, Gabata T, Higuchi R, Okamoto K, Hata J, Murata A, Kusachi S, Windsor JA, Supe AN, Lee S, Chen XP, Yamashita Y, Hirata K, Inui K, Sumiyama Y (2013) TG13: Updated Tokyo Guidelines for the management of acute cholangitis and cholecystitis. J Hepatobiliary Pancreat Sci 20 (1):1-7. doi:10.1007/s00534-012-0566-y

Xie G, Jiang J, Zhu P, Wang Z, Qiu J (2001) [Relationship between the dynamic expression of scavenger receptor on Kupffer cells and endotoxin-induced hepatic injury]. Zhonghua Gan Zang Bing Za Zhi 9 (2):117-119

Yu H, Ha T, Liu L, Wang X, Gao M, Kelley J, Kao R, Williams D, Li C (2012) Scavenger receptor A (SR-A) is required for LPS-induced TLR4 mediated NF-kappaB activation in macrophages. Biochim Biophys Acta 1823 (7):1192-1198. doi:10.1016/j.bbamcr.2012.05.004

Zani IA, Stephen SL, Mughal NA, Russell D, Homer-Vanniasinkam S, Wheatcroft SB, Ponnambalam S (2015) Scavenger receptor structure and function in health and disease. Cells 4 (2):178-201. doi:10.3390/cells4020178

Zhang CB, Tang YC, Xu XJ, Guo SX, Wang HZ (2015) Hydrogen gas inhalation protects against liver ischemia/reperfusion injury by activating the NF-kappaB signaling pathway. Exp Ther Med 9 (6):2114-2120. doi:10.3892/etm.2015.2385

Zhao W, Sun Z, Wang S, Li Z, Zheng L (2015) Wnt1 Participates in Inflammation Induced by Lipopolysaccharide Through Upregulating Scavenger Receptor A and NF-kB. Inflammation. doi:10.1007/s10753-015-0147-8

Zhou Y, Chen K, He L, Xia Y, Dai W, Wang F, Li J, Li S, Liu T, Zheng Y, Wang J, Lu W, Yin Q, Lu J, Teng H, Guo C (2015) The Protective Effect of Resveratrol on Concanavalin-A-Induced Acute Hepatic Injury in Mice. Gastroenterol Res Pract 2015:506390. doi:10.1155/2015/506390

Zuo D, Yu X, Guo C, Wang H, Qian J, Yi H, Lu X, Lv ZP, Subjeck JR, Zhou H, Sanyal AJ, Chen Z, Wang XY (2013) Scavenger receptor A restrains T-cell activation and protects against concanavalin A-induced hepatic injury. Hepatology 57 (1):228-238. doi:10.1002/hep.25983

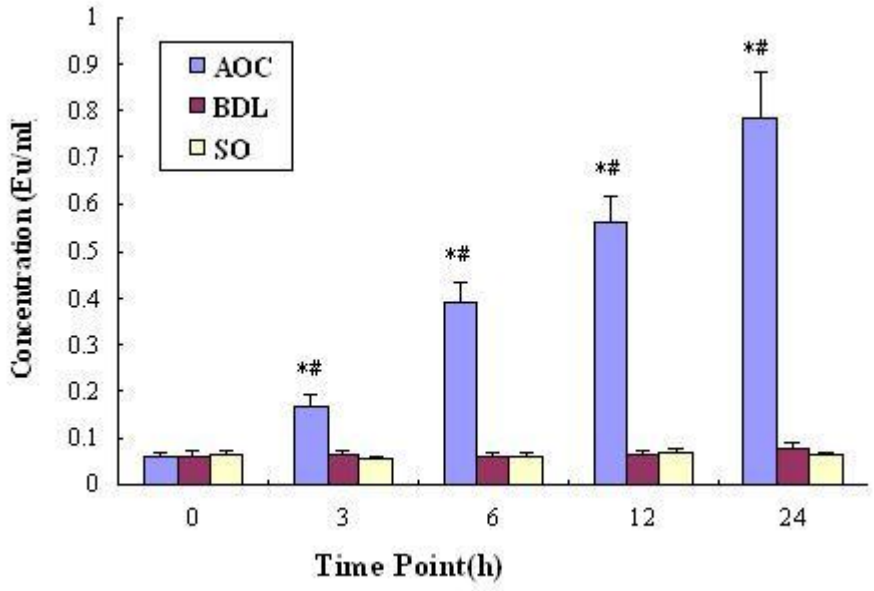

Figure 1-1 The comparison of plasma endotoxin concentration in various time points. Compared with Bile Duct Ligation (BDL) and Sham Operation (SO) groups, * P<0.01; Compared with same group, # P<0.01.

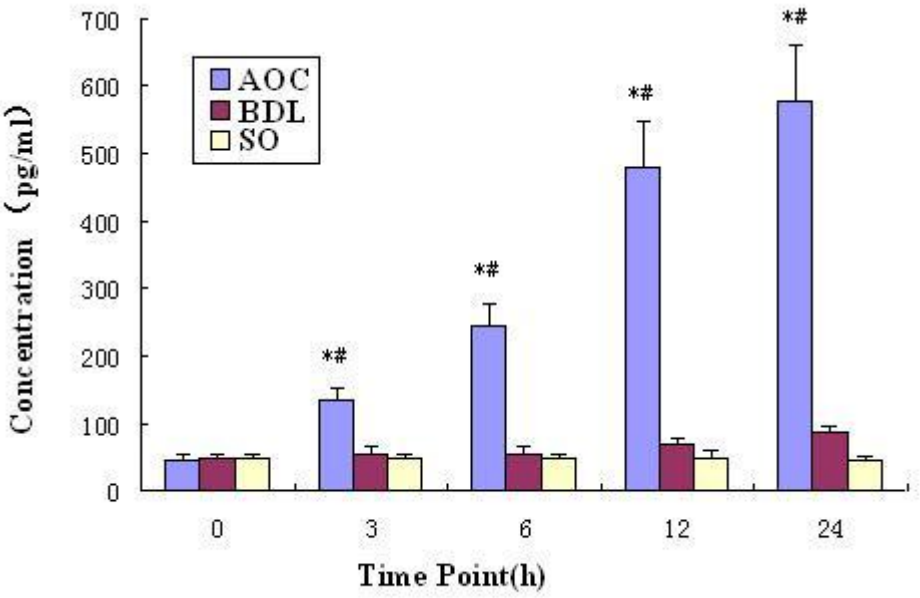

Figure 2-1 The comparison of plasma TNF-α concentration in various time points. Compared with Bile Duct Ligation (BDL) and Sham Operation (SO) groups, * P<0.01; Compared with same group, # P<0.01.

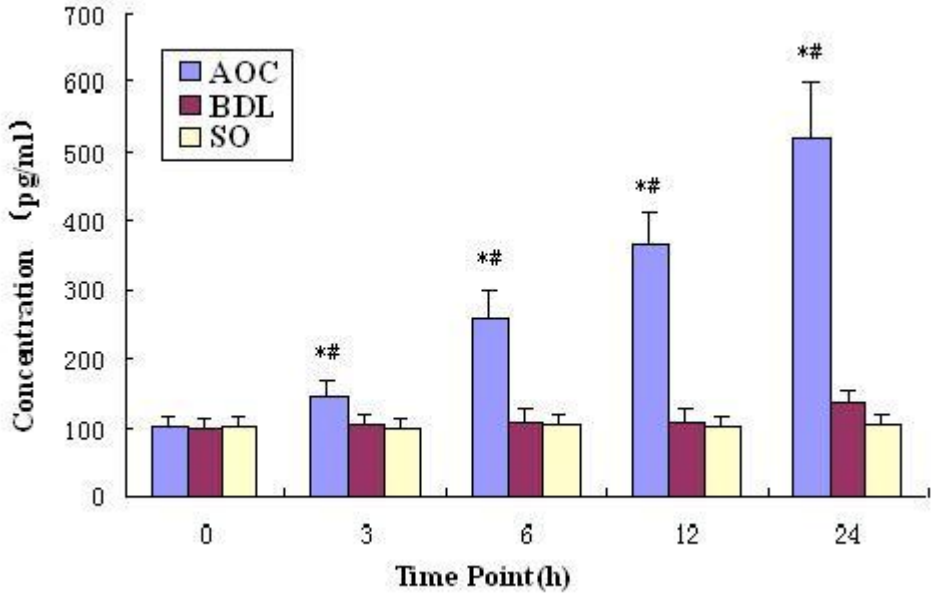

Fig 2-2 The comparison of plasma IL-6 concentration in various time points. Compared with Bile Duct Ligation (BDL) and Sham Group (SO) groups, * P<0.01; Compared with same group, # P<0.01.

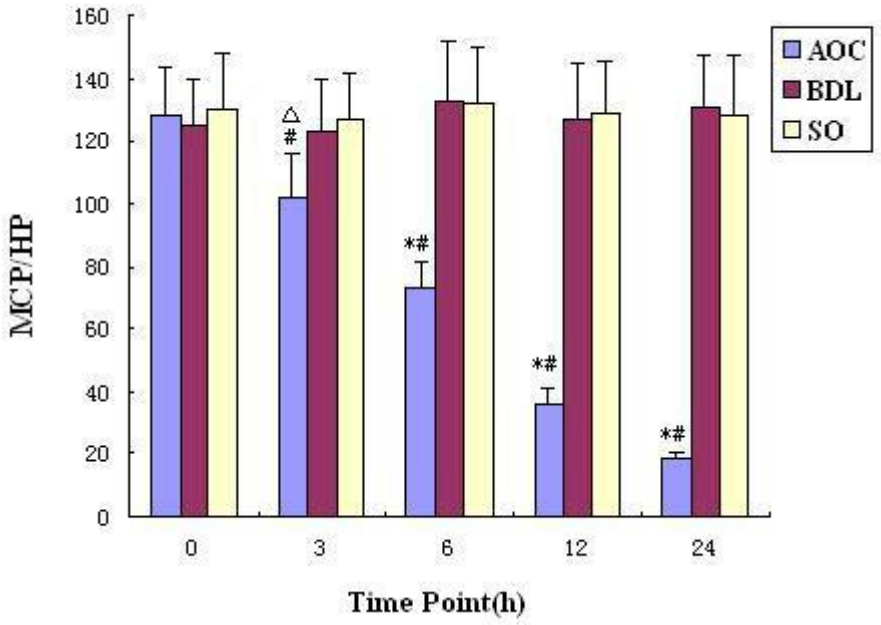

Figure 3-1 The comparison of Scavenger Receptor A (SRA) expression in various time points（IHC） mcp/HP. Compared among Acute Obstructive Cholangitis (AOC) group, Bile Duct Ligation (BDL) group and Sham Operation (SO) groups, * $P<0.01$, △ $P<0.05$; Compared with same group, # $P<0.01$.

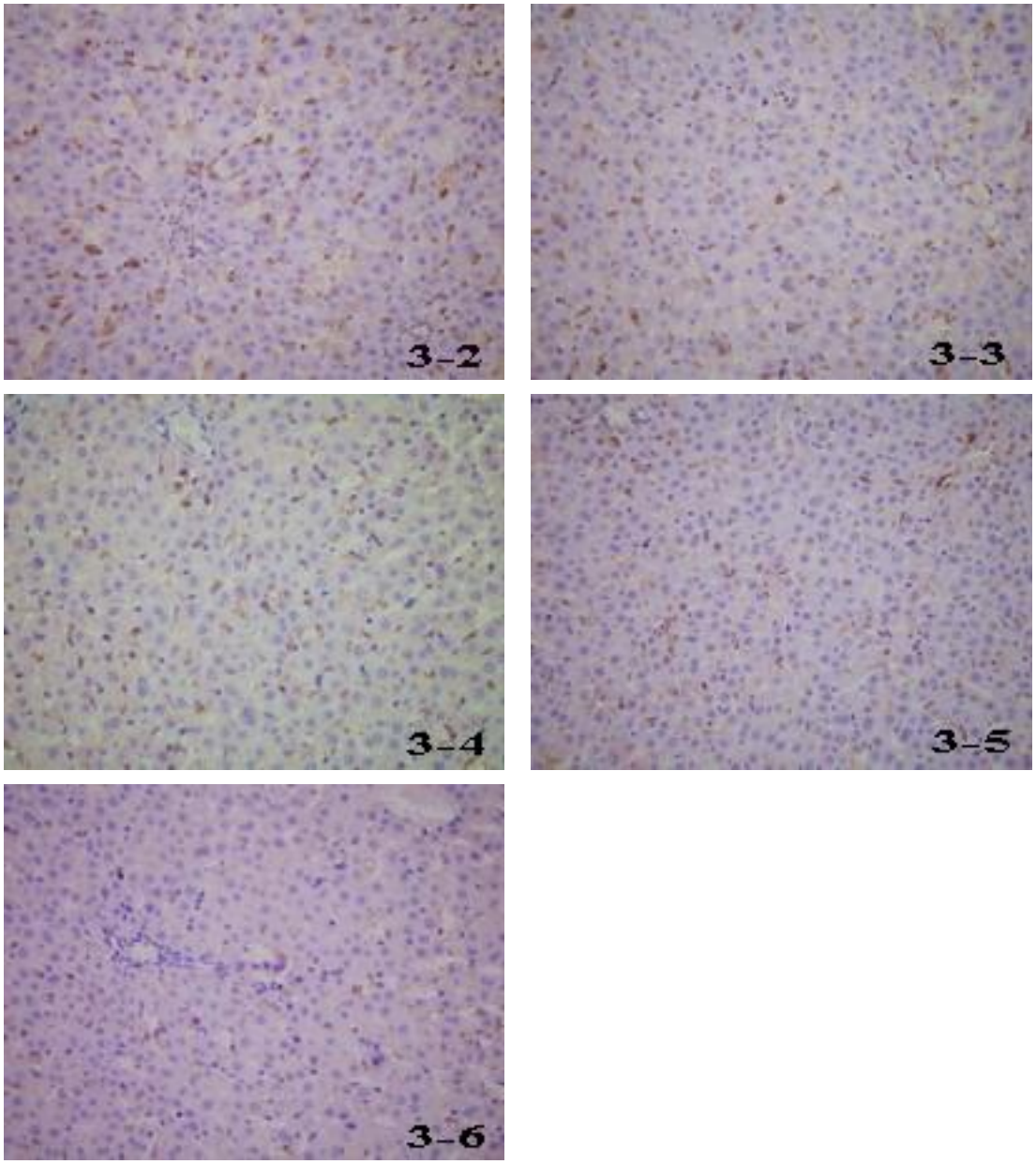

Figure 3-2 Kupffer cells' SRA expression in Acute Obstructive Cholangitis (AOC) groups at 0 hours (SP × 400). The brown positive products of SRA expression are mainly located on the surface of Kupffer cells and diffusely distributed in liver tissue.

Figure 3-3 to Figure 3-6 Kupffer Cells' SRA expression in liver tissue in Acute Obstructive Cholangitis (AOC) group at 3，6，12，24 hours (SP × 400). SRA expression products reduce with prolonged experimental time.

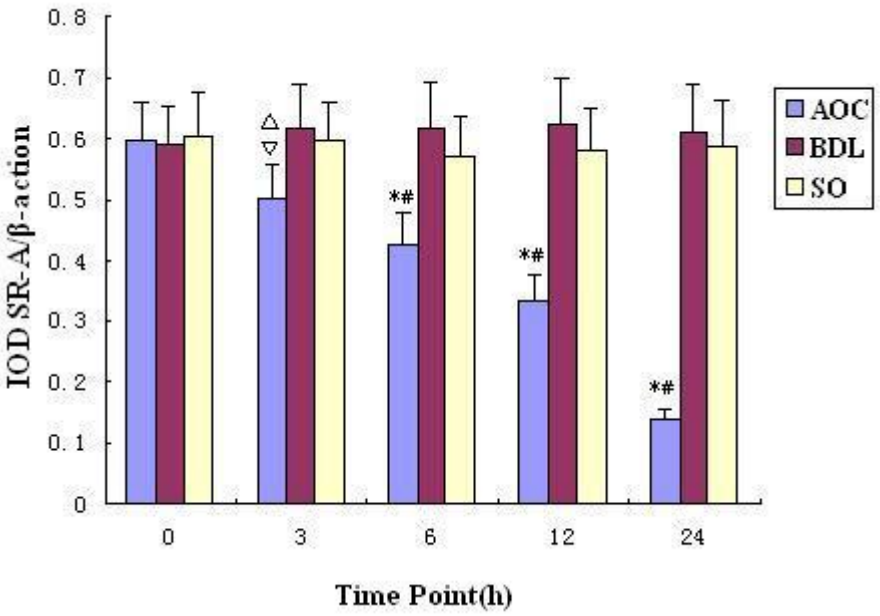

Figure 4-1 Relative expression of Scavenger Receptor A (SRA) mRNA in liver tissue in various time points (IODSR-A/IODβ-action) Compared among Acute Obstructive Cholangitis (AOC) group, Bile Duct Ligation (BDL) group and Sham Operation (SO) group, * $P<0.01$, △ $P<0.05$; Compared with same group, # $P<0.01$, ▽ $P<0.05$.

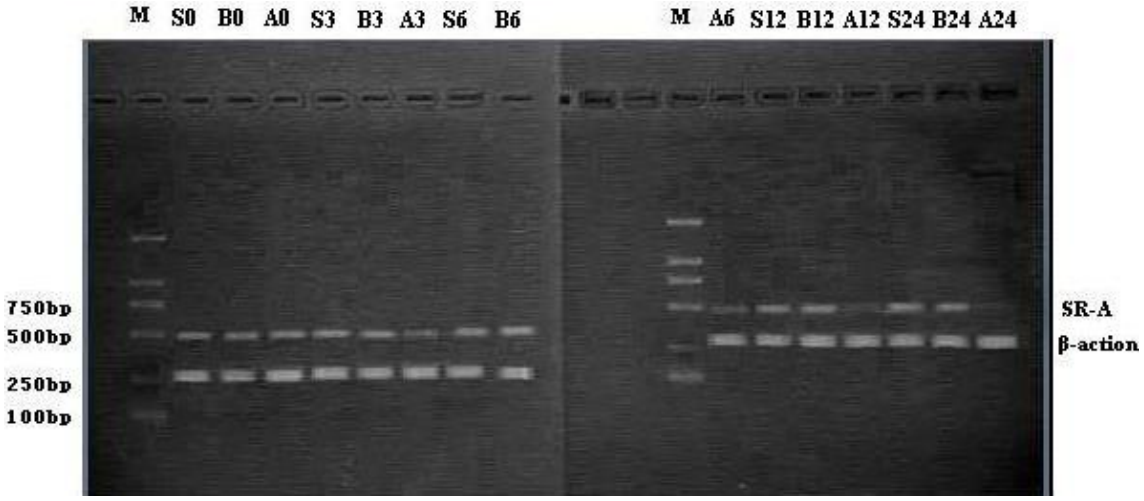

Figure 4-2 Relative expression of SRA mRNA in various time points by RT-PCR.
SRA mRNA expression in Acute Obstructive Cholangitis (AOC) group progressively decrease with prolonged experimental time.
M: marker; A, B, S: AOC, BDL, Sham Operation (SO) group, subsequent number stand for time point.

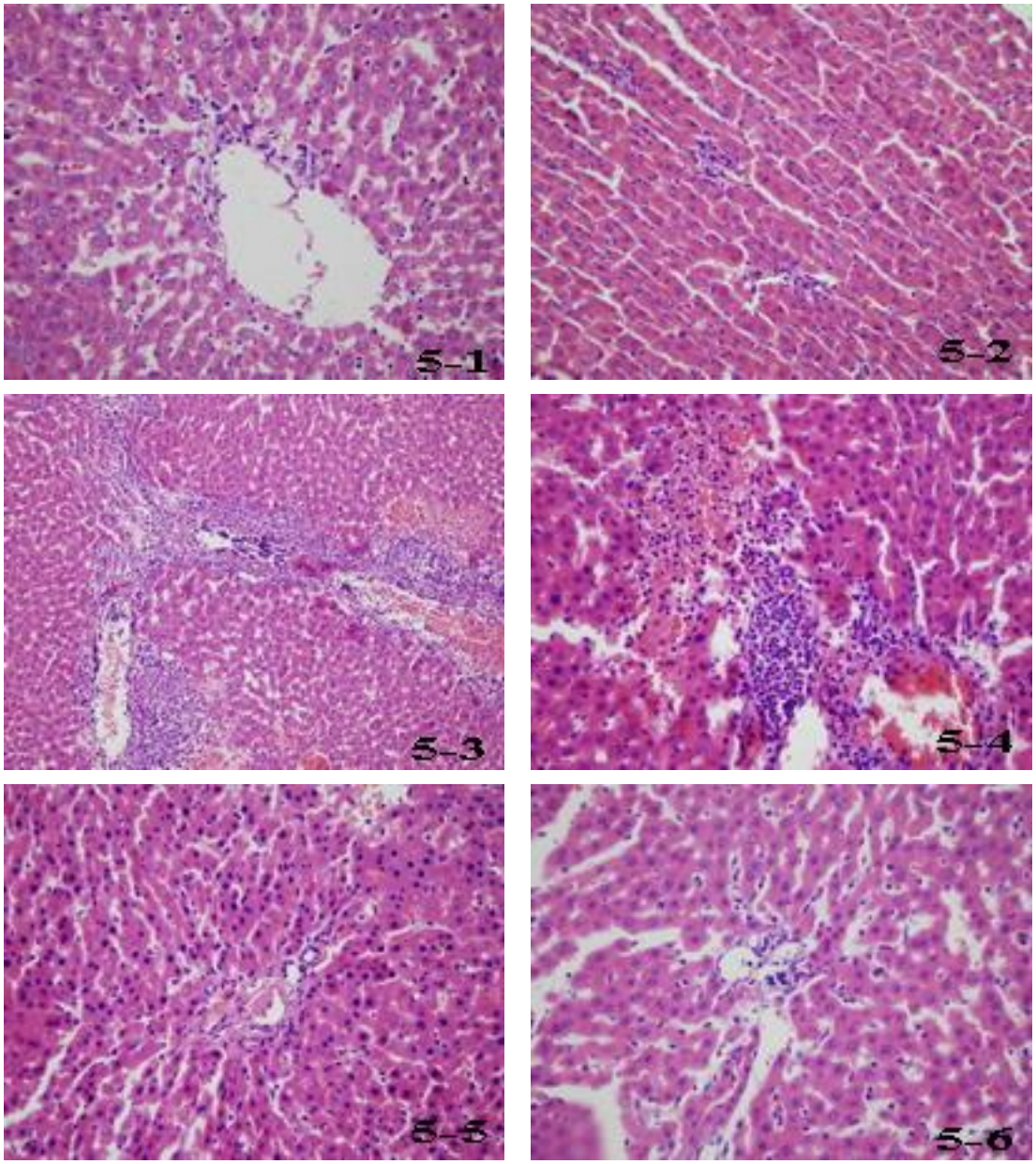

Figure 5-1~5-4 Liver tissue in Acute Obstructive Cholangitis (AOC) group at 3,6,12 and24 hours(HE ×400)

Many inflammatory infiltration mainly consisting of neutrophil and expansion of central veins are found at 3h. Focal inflammation are found in liver parenchyma at 6h. Obvious expansion of portal area, massive mixing inflammatory cells infiltration are found at 12h. Large-area hepatocyte degeneration and necrosis are found at 24h.

Figure 5-5 Liver tissue in Bile Duct Ligation (BDL) group (HE ×400), Manipulus Inflammatory cells infiltration are found in portal area.

Figure 5-6 Liver tissue in Sham Operation (SO) group (HE ×400), No obvious histopathological changes are found in liver tissue.

SUMMARY OF THESIS

Cholangitis is the inflammation of biliary ducts which usually begins extra-hepatic and easily spreads intra-hepatic, causing bacteremia. Acute obstruction of biliary tract with the presence of pus is severe form of cholangitis know as Acute Obstructive Suppurative Cholangitis (AOSC) which has very high mortality. It is experienced that the Charcot's triad and Reynold pentad are difficult to use in clinical practice for the management of cholangitis.

From our study, the expression of HMGB1 mRNA and HMGB1 Proteins are found higher in AOSC patients and it gradually decreased to normal level after the treatment of the disease suggesting HMGB1 as novel biomarker for AOSC. Also, the mechanism of HMGB1 with the patients of AOSC remain unclear and some work remain to be done to control AOSC-induced sepsis through anti-HMGB1 therapy and this needs to be further researched.

In rat AOC model, the expression of SRA from liver Kupffer cells decreased gradually with increasing plasma endotoxin leading to progressive liver injury of hepatocyte necrosis suggesting the importance of SRA in the defense mechanism during AOC.

Hence, we suggest High-mobility group box 1 and scavenger receptor A can be novel applications in the management of patients with cholangitis.

PUBLICATIONS DURING MY DOCTORAL PROGRAM

- ✓ Expression of Scavenger Receptor A in Rat's Liver Tissue during Acute Obstructive Cholangitis and Its Significant. SpringerPlus (Major Revision). First Author
- ✓ HMGB1 is Highly Expressed in the Patients with Acute Obstructive Suppurative Cholangitis-induced Sepsis. J Inflamm Res. 2015;8:71-7. First Author

AWARDS DURING MY DOCTORAL PROGRAM

- ✓ Awarded Excellent International Student among all the International Students of China by China Scholarship Council in the year of 2015 with cash prize of RMB 30000.00
- ✓ Won the title of "Excellent Competitor" in Chinese Language Competition 2015 organized by Chongqing Education Commission at International Chinese Culture Festival
- ✓ Won the Chinese Government Scholarship for my Doctoral program at Chongqing Medical University in 2013.

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ORIGINAL RESEARCH

Role of high-mobility group box 1 in patients with acute obstructive suppurative cholangitis-induced sepsis

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Background: High-mobility group box 1 (HMGB1) is a proinflammatory cytokine that plays an active role during the pathogenesis of inflammatory processes. The primary aim of this study was to detect whether HMGB1 is involved in the pathogenesis of acute obstructive suppurative cholangitis (AOSC).

Methods: We collected peripheral blood samples from 23 patients with AOSC and 23 healthy volunteers who served as normal controls. All participants were tested for HMGB1 mRNA level, HMGB1 protein, tumor necrosis factor alpha (TNF-alpha), and interleukin 10 (IL-10). HMGB1 mRNA levels were tested using real-time polymerase chain reaction. HMGB1 protein expression was measured using Western blot. TNF-alpha and IL-10 were tested using enzyme-linked immunosorbent assay.

Results: The expression of HMGB1 mRNA and HMGB1 protein was higher in the AOSC group than in the normal controls ($P<0.01$), and the levels gradually decreased to normal after treatment of the disease ($P<0.01$). The content of TNF-alpha and IL-10 in peripheral blood of patients with AOSC was significantly higher than that of normal controls ($P<0.01$) but decreased to normal levels after the necessary treatment ($P<0.01$).

Conclusion: The levels of HMGB1 mRNA and HMGB1 protein were elevated in patients with AOSC, which may play an important role in the inflammation of the bile duct and appears to be associated with the development of sepsis. This suggests the importance of monitoring HMGB1 levels in the management of AOSC-induced sepsis.

Keywords: AOSC, HMGB1, interleukin 10, TNF-alpha, sepsis, inflammation

Introduction

High-mobility group box 1 (HMGB1) is a chromatin-binding protein that is normally located predominantly inside the nucleus of cells. It is primarily responsible for transcription and growth factors. Recent studies identified HMGB1 as a mediator of inflammation when it is released into the extracellular environment, and it has been demonstrated to be a key factor for the development of acute inflammation. In response to infection, HMGB1 acts as an important proinflammatory cytokine that may lead to damage of cells and tissues. This process may progress to metabolic acidosis, multiorgan dysfunction syndrome, and sepsis. It is universally accepted that sepsis, which is fatal, is mediated by inflammatory mediators such as tumor necrosis factor alpha (TNF-alpha), interleukin 10 (IL-10), HMGB1, and so forth. All these factors have important actions in the startup, maintenance, and resolution of inflammation. TNF-alpha and IL-10 are early inflammatory factors that occur in the early phase of inflammation, during the onset of sepsis, and even a minimal

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delay in the therapeutic method directed against the early inflammatory factors make the management of this disease difficult for clinicians. Serum HMGB1 was significantly elevated from 8 to 72 hours after endotoxin exposure, in comparison with early inflammatory mediators TNF-alpha and interleukin.[1,2] Treatment with delayed administration of anti-HMGB1 antibodies,[3] a box of HMGB1,[3] and ethyl pyruvate beginning as late as the disappearance of plasma TNF-alpha and interleukin significantly increases patient survival.[4] Thus, targeting HMGB1 in the treatment of sepsis has a wider role than targeting early inflammatory cytokines such as TNF-alpha and IL-10 in the management of sepsis, and recent studies have reported several agents that target HMGB1 for the treatment of sepsis.[5,6]

Cholangitis is the inflammation of biliary ducts, which usually begins extrahepatically and easily spreads intrahepatically, causing bacteremia. It was first described as fever, abdominal pain, and jaundice by Charcot in 1877 and was known as the "Charcot Triad." Later, in 1959, Reynolds added mental confusion and septic shock to the description, and it became known as "Reynolds Pentad." The obstruction of biliary ducts and the presence of superposing bacterial infection are common features in cholangitis. Obstruction of the biliary tract is primarily caused by choledocholithiasis. Moreover, malignancy, benign strictures, and interventions to the biliary duct may also be the cause of biliary obstruction. Acute obstruction of the biliary tract with the presence of pus is a severe form of cholangitis known as acute obstructive suppurative cholangitis (AOSC), which has very high mortality if the drainage is delayed. Charcot's Triad and Reynold's Pentad are difficult to use in clinical practice for the management of cholangitis. The recently released "TG13: Updated Tokyo Guidelines for the management of acute cholangitis and cholecystitis," published in 2013, have not been evaluated or revised.[7] In our previous study, we found that nuclear factor kB, a very early mediator of inflammation, was markedly increased in patients with severe acute cholangitis and that the degree of nuclear factor kB correlates with the degree of severity of the cholangitis.[8] The degree of nuclear factor kB also correlates to clinical outcome. In another study of patients with cholangitis, we observed that TREM-1, which is also an early inflammatory cytokine, provided a useful early indicator for sepsis.[9] However, little to no research exits regarding the late inflammatory mediators in the patients with AOSC or sepsis induced by AOSC. Recent studies have reported associations between HMGB1 and inflammation.[10–12] Previously, we found that HMGB1 represents a potentially valuable marker for the diagnosis of acute appendicitis.[13] However, the correlation between HMGB1 and AOSC pathogenesis is not yet fully understood. Our objective in the current study was to determine the role of HMGB1 in the inflammatory process of AOSC.

Materials and methods

All experimental protocols described in this study were approved by the research ethics review committee of the Second Affiliated Hospital of Chongqing Medical University and comply with the Chinese government guidelines. A total of 23 AOSC-induced patients with sepsis admitted to the Department of Hepatobiliary Surgery, the Second Affiliated Hospital of Chongqing Medical University, People's Republic of China, between November 2010 and June 2011 were randomly enrolled in the prospective study. During that period, 23 healthy individuals were also randomly selected from the Physical Examination Center of the Second Affiliated Hospital, Chongqing Medical University, People's Republic of China, to be used as a control group. Written consent was obtained from all volunteers. All AOSC-induced patients with sepsis and controls were between the ages of 18 and 80 years and were ethnically similar (Han Chinese). The selection of AOSC-induced patients with sepsis was based on diagnostic criteria and severity assessment of acute cholangitis, using the Tokyo Guidelines[14] and International Sepsis Definitions Conference 2003.[15] The patients involved in this study underwent biliary drainage via endoscopic retrograde cholangiopancreatography, or percutaneous transhepatic catheter drainage, or endoscopic ultrasound guided drainage, or open surgical drainage.

Peripheral blood samples of each AOSC-induced patient with sepsis were obtained predrainage, 12 hours postdrainage, and 24 hours postdrainage. In addition, peripheral blood samples from the individuals in the control group were obtained after overnight fasting.

Each sample was collected in 5 mL tubes, one with and one without heparin. The peripheral blood mononuclear cells (PBMCs) were immediately separated from the samples with heparin, using a lymphocyte separating medium and a density gradient centrifuge. After the blood clotted in the tube without heparin, the serum was separated and taken out of the tube and then stored at −70°C.

mRNA measurement using real-time polymerase chain reaction

PBMCs were isolated from the blood samples using a lymphocyte separation medium (Bosoter, People's Republic

of China), a Ficoll density gradient isolating lymphocytes by centrifuging. The single-step technique of acid guanidinium thiocyanate chloroform extraction was used for total RNA extraction according to the instructions provided by the manufacturer. We determined the concentration of purified total RNA spectrophotometrically at a wavelength of 280 nm. The HMGB1 mRNA and β-actin levels in blood samples were quantified in duplicate, using real-time polymerase chain reaction. We removed any potential DNA contamination using DNase I. We used 1 μg total RNA from each sample for reverse transcription with an oligo (dT) and Super Script II reverse transcriptase to generate first-strand cDNA. Thermal cycling conditions were as follows: 2 minutes at 94°C, followed by 35 cycles at 94°C for 30 seconds and at 60°C for 3 minutes on a Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The expression was normalized to β-actin mRNA content. Sequences of the human primers for polymerase chain reaction were as follows: HMGB1 Bbox (127 bp): 5′-GCG GAC AAG GCC CGT TA-3′ (sense), 5′-AGA GGA AGA AGG CCG AAG GA-3′ (antisense); β-actin (255 bp): 5′-CAA AGA CCT GTA CGC CAA CA-3′ (sense), 5′-GAA GCA TTT GCG GTG GAC-3′ (antisense).

HMGB1 protein measurement using Western blot analysis

PBMCs were isolated from the blood samples as previously described. The difference between HMGB1 protein expression levels was analyzed using a Western blot. Protein was extracted from PBMCs by homogenizing samples in a cell lysis buffer. Proteins were quantified using a Nanodrop 1000 (Thermo Scientific, Wilmington, DE, USA). Equal amounts of protein were loaded in each well for electrophoresis in 10% sodium dodecyl sulfate-polyacrylamide gels and then transferred to polyvinylidene fluoride micropoporous membranes (Millipore Corporation, Billerica, MA). The membrane containing the transferred proteins was blocked with 5% bovine serum albumin and then incubated with rabbit anti-HMGB1 polyclonal antibody (1:250; BD Pharmingen, San Diego CA), followed by an incubation with horseradish peroxidase-linked secondary antibody (1:1,000; Golden Bridge, Beijing, People's Republic of China). For standardization and comparison, the membranes were also hybridized to a primary anti β-actin antibody (1:1,000; Santa Cruz Technology, Santa Cruz, CA, USA) or antihistine H 3.1 antibody (1:1,000; SAB, Pearland, TX, USA). The bands appearing on film were analyzed with Gene Tools software (Syngene, Frederick, MD, USA).

Enzyme-linked immunosorbent assay

The concentration of TNF-alpha and IL-10 in the serum was detected using commercially available enzyme-linked immunosorbent assay (ELISA) kits (R and D Systems, Minneapolis, MN, USA), following the manufacturer's instructions.

These three statistical analyses were completed using SPSS19.0 software for Windows (SPSS, Chicago, IL, USA). Data are presented as the mean ± SEM unless otherwise indicated. A Kruskal-Wallis analysis was performed to ascertain that there is an overall difference, and a Mann-Whitney U test was used to test differences between the groups. P values <0.05 were considered statically significant. P values <0.01 were considered notably statistically significant.

Results

Association of HMGB1 mRNA expression in PBMCs of AOSC patients

Total RNA was extracted from PBMCs, and the HMGB1 mRNA was amplified via real-time polymerase chain reaction to detect the expression of HMGB1 mRNA in PBMCs of patients with AOSC (Figure 1). As shown in Figure 3, the level of HMGB1 mRNA in PBMCs was 0.82±0.04 in patients

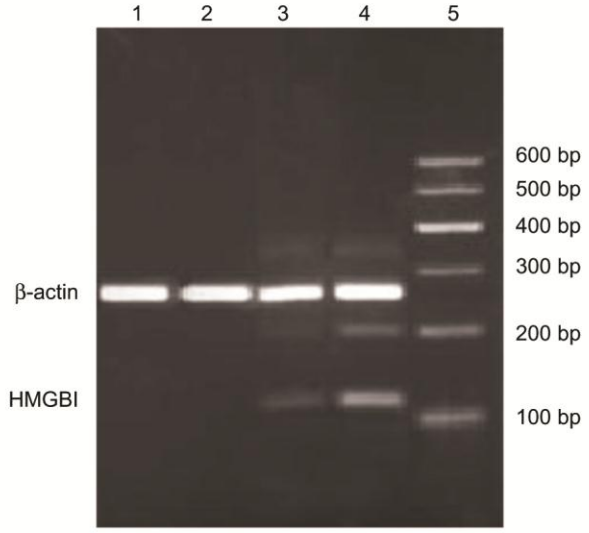

Figure 1 Expression of high-mobility group box 1 mRNA in peripheral blood mononuclear cells by real-time polymerase chain reaction analysis.

Notes: Total RNA was obtained from peripheral blood mononuclear cells of the control group and from acute obstructive suppurative cholangitis-induced sepsis patients (before the drainage, 12 hours after the drainage, and 24 hours after the drainage, respectively). Electrophoresis of high-mobility group box 1 gene fragment amplification product is shown. Lane 1 represents polymerase chain reaction product of control group, lane 2 represents polymerase chain reaction product of AOSC-induced sepsis group 24 hours after the drainage, lane 3 represents polymerase chain reaction product of AOSC-induced sepsis group 12 hours after the drainage, lane 4 represents polymerase chain reaction product of AOSC-induced sepsis group before the drainage and lane 5 represents DL2000 marker.

Abbreviations: AOSC, acute obstructive suppurative cholangitis; HMGB1, high-mobility group box 1.

with AOSC, which was significantly higher (62%) than that of healthy donors (0.31±0.06; $P<0.01$). We also found that the level of HMGB1 mRNA gradually decreased to 0.4±0.05 and 0.38±0.07 ($P<0.01$) after 12 and 24 hours postdrainage, respectively. The 12-hour postdrainage level of HMGB1 mRNA was decreased by 32% relative to the predrainage level.

Association of HMGB1 protein in PBMCs of patients with AOSC

The HMGB1 protein levels in PBMCs of both groups, the patients with AOSC, and the healthy donors were tested using a Western blot analysis (Figure 2). As shown in Figure 3, we found that the level of the HMGB1 protein in patients with AOSC was 0.81±0.03, which was significant and 57% higher than that of 0.35±0.05 ($P<0.01$) seen in healthy donors. The level of HMGB1 proteins gradually decreased to 0.56±0.05 and 0.42±0.07 ($P<0.01$) after 12 and 24 hours postdrainage, respectively. The 12-hour postdrainage level of HMGB1 protein decreased by 37% compared with the predrainage level.

Association of TNF-alpha and IL-10 expression in the serum of patients with AOSC

The level of TN-alpha and IL-10 proteins in the serum of each sample from patients with AOSC and healthy donors was determined using commercially available ELISA kits. We found that TNF-alpha and IL-10 were significantly higher in the patients than in healthy donors and that they gradually decreased after biliary passage drainage (Figure 4). The average predrainage value of TNF-alpha was 469±35 pg/mL, which decreased to 243±26 pg/mL and 132±47 pg/mL after 12 and 24 hours postdrainage, respectively; it was 102±16 pg/mL in the healthy controls. In the same regard, the average

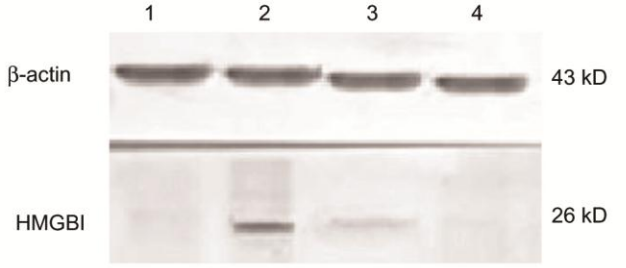

Figure 2 Western blotting analysis of high-mobility group box 1 protein expression in peripheral blood mononuclear cells.
Notes: Western blotting was performed to detect protein level of high-mobility group box 1. Total protein was extracted from peripheral blood mononuclear cells. Lane 1 represents the control, lane 2 represents preoperation, lane 3 represents postoperation 12 hours, and lane 4 represents postoperation 24 hours.
Abbreviation: HMGB1, high-mobility group box 1.

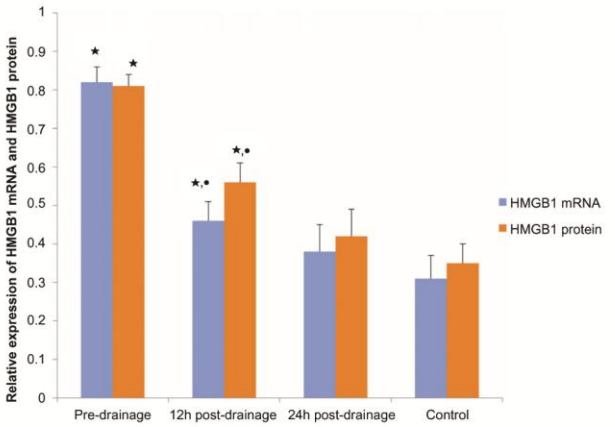

Figure 3 Comparison of high-mobility group box 1 (HMGB1) mRNA and high-mobility group box 1 protein expression among patients with acute obstructive suppurative cholangitis and controls (n=23).
Notes: *Compared with control, $P<0.01$; •compared with predrainage, $P<0.01$.

predrainage value of IL-10 was 33.369±6.443 pg/mL, which decreased to 20.562±5.630 pg/mL and 13.742±3.374 pg/mL after 12 and 24 hours postdrainage, respectively; it was 11.490±1.088 pg/mL in healthy controls (Figure 5).

Discussion

Acute cholangitis is a systemic infectious disease characterized by acute inflammation and biliary tract infection. Acute cholangitis occurs as a result of bacterial colonization within the biliary tract, which increases the internal pressure of the biliary tract and causes a backflow of bacteria and endotoxins within the circulatory system.[16,17] AOSC is a severe form of cholangitis in which pus collects in the biliary tract. Drainage of the biliary tract should be performed as soon as possible in patients with AOSC; otherwise, translocation of

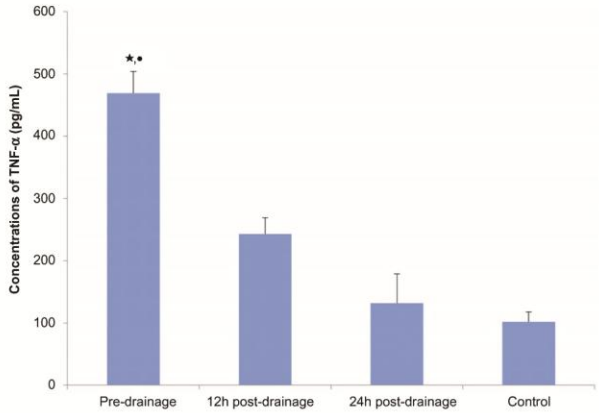

Figure 4 The analysis of tumor necrosis factor alpha by enzyme-linked immunosorbent assay (ELISA) in serum of operated and healthy controls.
Notes: Tumor necrosis factor alpha (TNF-α) was quantitated using an ELISA kit. Data are expressed as median for each sample. Experiments were performed at least three times, getting similar results. *Compared with control, $P<0.01$; •compared with 12 hours postdrainage, $P<0.01$.

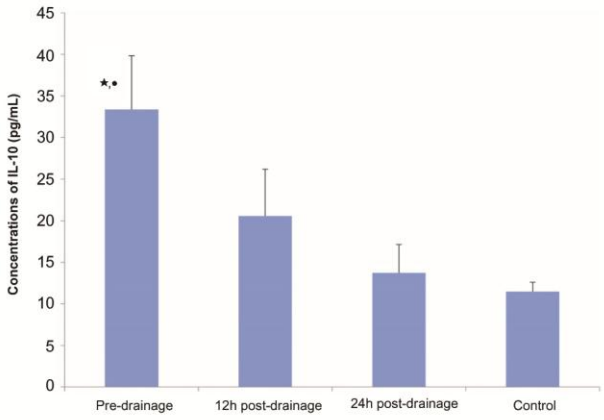

Figure 5 The analysis of interleukin 10 (IL-10) by enzyme-linked immunosorbent assay (ELISA) in serum of operated and healthy controls.
Notes: Tumor necrosis factor alpha was quantitated using ELISA kit. Data are expressed as median for each sample. Experiments were performed at least three times getting similar results. *Compared with control, $P<0.01$; •compared with 12 hours postdrainage, $P<0.01$.

bacteria into the bloodstream results in septicemia, an often fatal complication of acute cholangitis that induces severe organ damage and high mortality. There was no appropriate guideline for the diagnosis and management of acute cholangitis before the TG13: Updated Tokyo Guidelines for the management of acute cholangitis and cholecystitis.[7] The symptoms and signs described as Charcot's Triad and Reynold's Pentad are difficult for physicians to use in diagnostic and management strategies in clinical settings. The newly published TG13 is considered to have better diagnostic and management capacity. However, TG13 lacks sufficient feedback and revisions. Within 1 year, several studies found that the diagnostic criteria of TG13 fail to identify severe cases of cholangitis that require emergency biliary drainage.[18] Hence, the management strategy of TG13 may not be especially specific to AOSC, which is a severe form of cholangitis in which emergency biliary drainage is a necessity. Further study of the diagnosis and management of AOSC is necessary. A marker to be used to diagnose AOSC-induced cholangitis would present a helpful step in the management of AOSC. Recently, several studies have found that HMGB1 is an important agent for the diagnosis and management of sepsis. The present study focused on the correlation of HMGB1 with cholangitis in patients with AOSC.

Many clinical and experimental studies have described the release of HMGB1 during inflammation.[13,19] It has been shown that HMGB1 is a mediator of inflammation when it is released extracellularly. The extracellular release of HMGB1 may occur either through active release by immune cells or passive secretion after cell death. Inside the nucleus, HMGB1 functions as a protein for binding chromatin and helps in the transcription process of genes.[20] Extracellular release of HMGB1 is a result of translocation of HMGB1 from the nucleus to the cytoplasm, which involves acetylation,[21] phosphorylation,[22] and methylation.[23] Extracellular HMGB1 then exerts biological expression via its receptors. HMGB1 signaling is mediated by several receptors, including Receptor for Advanced Glycation Endproduct and Toll-like receptors.[24,25] During infection, endotoxins are produced by bacteria that activate monocytes and macrophages such as TNF-alpha, IL-10, and so on. These cytokines promote HMGB1 translocation from the nucleus into the cytoplasm and further enter into transcription processes by themselves.[5,26,27] These produced HMGB1 proteins, act as cytokines, which further induce the stimulation of other inflammatory agents, resulting in a worsening in the severity of inflammation.

Our study demonstrated that the expression levels of TNF-alpha, IL-10, and HMGB1 were significantly higher in patients with AOSC than in normal controls. After drainage, the level of HMGB1, TNF-alpha, and IL-10 in the patients with AOSC decreased as infection gradually decreased, indicating that with the exacerbation of AOSC, HMGB1 is positively correlated with the disease. Increased expression of TNF-alpha and IL-10 has also been observed in noninflammatory diseases, whereas increased expression of HMGB1 is specifically observed in the PBMCs and serum in patients with sepsis. Recent studies have also reported that the severity of the disease increases with the increase in the expression of HMGB1. In severely burned patients, it has been reported that the level of HMGB1 expression is significantly higher in deceased patients than in survivors.[28] HMGB1 has been studied for more than 4 decades, and in recent years, it has gained attention as a potential marker of sepsis. Our previous study demonstrated that HMGB1 expression may represent a novel marker for the diagnosis of acute appendicitis and also aids physicians in deciding the severity of the disease.[13] Because TNF-alpha and IL-10 are early markers of inflammation, whereas expression of HMGB1 is found in late stages of sepsis, HMGB1's significance in patients with sepsis can be higher than early inflammatory factors such as TNF-alpha and IL-10. At this time, several animal and clinical studies have also demonstrated a protective effect of anti-HMGB1 agents on sepsis. It should be noted that if sepsis is a result of HMGB1, there will be an adequate time interval to detect it and administer anti-HMGB1 for the therapeutic management of sepsis.

In patients with AOSC-induced sepsis, bacteria can easily enter the circulatory system because there is increased

intraductal pressure caused by blockage of the biliary tract. These bacteria produce endotoxins and proinflammatory agents. The produced proinflammatory agents activate monocytes and macrophages, which cause the release of HMGB1 in the extracellular environment. The released HMGB1 acts as a proinflammatory factor that worsens the inflammation and affects distal organs.[26,27] From this point of view, the increased level of HMGB1 could be directly correlated with the severity of ASOC-induced sepsis and, hence, can be useful as a diagnostic marker in patients with AOSC. In addition, controlling the expression of HMGB1 with an anti-HMGB1 agent may improve the efficacy of AOSC therapy by managing systemic inflammation and reducing organ injury by sepsis. However, there is a lack of clinical evidence of suppressing increased expression of HMGB1 in patients with sepsis, which requires further clarification by clinical trials results.

Conclusion

Our study demonstrated the increased expression of HMGB1 in peripheral blood mononuclear cells of AOSC-induced patients with sepsis, which may play an important role for the progression of sepsis with the patients with AOSC. The sample size of our study is small, and we did not include inflammation caused by other disorders that represent important parameters that affect sensitivity and specificity of HMBG1 in patients with AOSC. The mechanism of HMGB1 in patients with AOSC remains unclear and requires further study. This result is not designed to control AOSC-induced sepsis through anti-HMGB1 therapy, and this needs to be further researched. Although we have yet to complete further research to demonstrate the sensitivity and specificity of HMGB1 in the diagnosis and therapy for patients with AOSC, combined with other diagnostic markers, HMGB1 could provide a useful biomarker for AOSC-induced sepsis.

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Disclosure

The authors report no conflicts of interest in this work.

References

1. Wang H, Bloom O, Zhang M, et al. HMG-1 as a late mediator of endotoxin lethality in mice. *Science*. 1999;285(5425):248–251.
2. Yang H, Ochani M, Li J, et al. Reversing established sepsis with antagonists of endogenous high-mobility group box 1. *Proc Natl Acad Sci U S A*. 2004;101(1):296–301.
3. Li X, Wang LK, Wang LW, Han XQ, Yang F, Gong ZJ. Blockade of high-mobility group box-1 ameliorates acute on chronic liver failure in rats. *Inflammation Research: Official Journal of the European Histamine Research Society ... [et al.]*. Jul 2013;62(7):703–709.
4. Ulloa L, Ochani M, Yang H, et al. Ethyl pyruvate prevents lethality in mice with established lethal sepsis and systemic inflammation. *Proc Natl Acad Sci U S A*. 2002;99(19):12351–12356.
5. Fink MP. Neuropeptide modulators of high mobility group box 1 secretion as potential therapeutic agents for severe sepsis. *Am J Pathol*. 2008;172(5):1171–1173.
6. Yoo H, Ku SK, Baek YD, Bae JS. Anti-inflammatory effects of rutin on HMGB1-induced inflammatory responses in vitro and in vivo. *Inflammation Research: Official Journal of the European Histamine Research Society ... [et al.]*. Mar 2014;63(3):197–206.
7. Takada T, Strasberg SM, Solomkin JS, et al. TG13: Updated Tokyo Guidelines for the management of acute cholangitis and cholecystitis. *Journal of Hepato-Biliary-Pancreatic Sciences*. Jan 2013;20(1):1–7.
8. Gong JP, Liu CA, Wu CX, Li SW, Shi YJ, Li XH. Nuclear factor kB activity in patients with acute severe cholangitis. *World J Gastroenterol*. 2002;8(2):346–349.
9. Liao R, Liu Z, Wei S, Xu F, Chen Z, Gong J. Triggering receptor in myeloid cells (TREM-1) specific expression in peripheral blood mononuclear cells of sepsis patients with acute cholangitis. *Inflammation*. 2009;32(3):182–190.
10. El Gazzar M. HMGB1 modulates inflammatory responses in LPS-activated macrophages. *Inflammation Research: Official Journal of the European Histamine Research Society ... [et al.]*. Apr 2007;56(4):162–167.
11. Kao RL, Xu X, Xenocostas A, et al. Induction of acute lung inflammation in mice with hemorrhagic shock and resuscitation: role of HMGB1. *J Inflamm (Lond)*. 2014;11(1):30.
12. Hou C, Kong J, Liang Y, et al. HMGB1 contributes to allergen-induced airway remodeling in a murine model of chronic asthma by modulating airway inflammation and activating lung fibroblasts. *Cell Mol Immunol*. 2014;(Aug):25.
13. Wu C, Sun H, Wang H, et al. Evaluation of high mobility group box 1 protein as a presurgical diagnostic marker reflecting the severity of acute appendicitis. *Scand J Trauma Resusc Emerg Med*. 2012;20(1):61.
14. Wada K, Takada T, Kawarada Y, et al. Diagnostic criteria and severity assessment of acute cholangitis: tokyo Guidelines. *J Hepatobiliary Pancreat Surg*. 2007;14(1):52–58.
15. Levy MM, Fink MP, Marshall JC, et al; SCCM/ESICM/ACCP/ATS/SIS. 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference. *Crit Care Med*. 2003;31(4):1250–1256.
16. Kimura Y, Takada T, Strasberg SM, et al. TG13 current terminology, etiology, and epidemiology of acute cholangitis and cholecystitis. *Journal of Hepato-Biliary-Pancreatic Sciences*. Jan 2013;20(1):8–23.
17. Mosler P. Diagnosis and management of acute cholangitis. *Curr Gastroenterol Rep*. 2011;13(2):166–172.
18. Shinya S, Sasaki T, Yamashita Y, et al. Procalcitonin as a useful biomarker for determining the need to perform emergency biliary drainage in cases of acute cholangitis. *Journal of Hepato-Biliary-Pancreatic Sciences*. Oct 2014;21(10):777–785.
19. Wu CX, Sun H, Liu Q, Guo H, Gong JP. LPS induces HMGB1 relocation and release by activating the NF-κB-CBP signal transduction pathway in the murine macrophage-like cell line RAW264.7. *J Surg Res*. 2012;175(1):88–100.
20. Stott K, Tang GS, Lee KB, Thomas JO. Structure of a complex of tandem HMG boxes and DNA. *J Mol Biol*. 2006;360(1):90–104.
21. Li Y, Gan CP, Zhang S, et al. FIP200 is involved in murine pseudomonas infection by regulating HMGB1 intracellular translocation. *Cell Physiol Biochem*. 20133(6):1733–1744.

22. Ge X, Antoine DJ, Lu Y, et al. High mobility group box-1 (HMGB1) participates in the pathogenesis of alcoholic liver disease (ALD). *J Biol Chem*. 2014;289(33):22672–22691.
23. Wu F, Zhao ZH, Ding ST, Wu HH, Lu JJ. High mobility group box 1 protein is methylated and transported to cytoplasm in clear cell renal cell carcinoma. *Asian Pac J Cancer Prev*. 2013;14(10):5789–5795.
24. Park JS, Gamboni-Robertson F, He Q, et al. High mobility group box 1 protein interacts with multiple Toll-like receptors. *Am J Physiol Cell Physiol*. 2006;290(3):C917–C924.
25. Ivanov S, Dragoi AM, Wang X, et al. A novel role for HMGB1 in TLR9-mediated inflammatory responses to CpG-DNA. *Blood*. 2007;110(6):1970–1981.
26. Pisetsky DS. The expression of HMGB1 on microparticles released during cell activation and cell death in vitro and in vivo. *Mol Med*. 2014;20(1):158–163.
27. Rendon-Mitchell B, Ochani M, Li J, et al. IFN-gamma induces high mobility group box 1 protein release partly through a TNF-dependent mechanism. *J Immunol*. 2003;170(7):3890–3897.
28. Huang LF, Yao YM, Dong N, Yu Y, He LX, Sheng ZY. Association of high mobility group box-1 protein levels with sepsis and outcome of severely burned patients. *Cytokine*. 2011;53(1):29–34.