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Original Article

Effects of bone marrow MSCs transfected with sRAGE on the intervention of HMGB1 induced immuno-inflammatory reaction

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Abstract: Background: High mobility group box chromosomal protein 1 (HMGB1) is an important proinflammatory molecule in many inflammatory disorders, but little is known about its role in acute liver failure (ALF). In this study, we determined the plasma and hepatic tissue levels of HMGB1 in a d-galactosamine-induced rat ALF model and investigated the effect of soluble receptor for advanced glycation end products (sRAGE) on ALF successfully. Methods: Male Sprague-Dawley rats were divided into five groups randomly. Group A (Control group, n=20) received administrated saline via peritoneal cavity. Group B (ALF group, n=20) induced by d-galactosamine (0.6 g/kg) via peritoneal cavity. Group C (HMGB1 group, n=20) were treated with HMGB1 recombination protein (200 μg/kg) via penile vein after ALF model induced. Group D (sRAGE group, n=20) received administrated sRAGE recombination protein (400 μg/kg) via penile vein after ALF model induced. Group E (sRAGE-MSC group, n=20) received 3×10^6 MSC transplantation which could maintain a stable expression of sRAGE via penile vein after ALF model induced. Liver function, level of cytokines and liver pathological changes were measured. Results: We determined that the plasma levels and hepatic tissue levels of HMGB1 were significant increased in ALF model ($P<0.05$). SRAGE group and sRAGE-MSC group could significantly prolong ALF rat survival time, as well as improve its liver functions, inflammatory cytokines level and hepatocytes necrosis. Conclusion: SRAGE as a ligand decoy has illustrated largely beneficial effects on reducing immuno-inflammatory response, which holds promise for the identification of potential therapeutic targets and/or biomarkers of RAGE activity in ALF.

Keywords: Acute liver failure, bioartificial livers, high mobility group box chromosomal protein 1, soluble receptor for advanced glycation end products, Immuno-inflammatory reaction

Introduction

Acute liver failure (ALF) is a clinical syndrome characterized by progressive and massive hepatocellular necrosis [1]. The essence is that acute liver injury with hepatocellular necrosis was mainly caused by viral hepatitis or alcohol intake. Despite of the recent therapeutic advances, ALF remains a serious clinical condition that is associated with a high mortality rate. Although liver transplantation is sometimes the only effective treatment for ALF [2], the availability of both cadaveric and living donor organ is limited.

Recent developments in tissue engineering techniques have made it possible to utilize isolated hepatocytes for the configuration of bioartificial livers (BAL) [3-5]. It is generally anticipated that functional extracorporeal liver support systems could perform a wide spectrum of liver functions found in the normal liver. Our previous studies demonstrated for the first time that cell-matrix has synergic effects on the preservation of hepatic morphology and function in the co-culture of porcine hepatocytes with mesenchymal stem cells in vitro, which could represent a promising tool for tissue engineering, cell biology, and bioartificial liver devices [6]. Co-cultured

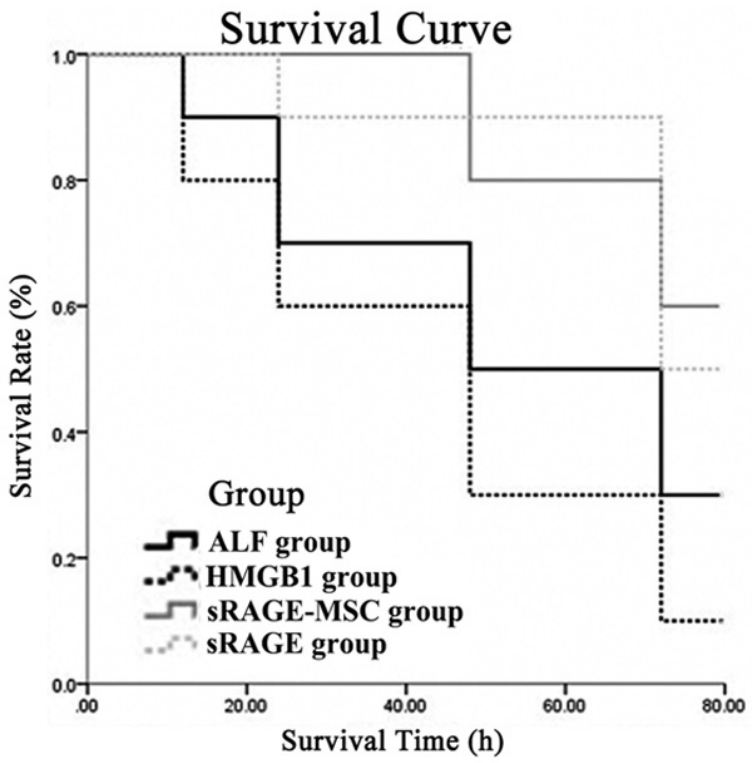

Figure 1. Survival of each group was recorded every 12 h, For normal group, ALF model group, rHMGB1 group, sRAGE group and GFP/sRAGE-transfected MSCs group, overall survival are 100%, 30%, 10%, 60% and 50%, respectively (72 h).

primary hepatocytes gain access to environment similar to that of normal human body, which help stabilize their structure and exert their biological function [7, 8]. This may provide insights into the current dilemma that bio-artificial liver cells dedifferentiate rapidly and are of limited liver support function. Therefore, BAL would serve as a bridge to liver transplantation or regeneration, which is needed to reduce the morbidity and mortality caused by ALF [9]. Until now, BAL support system is still not routinely used in clinical treatment. One of the essential issues is that the BALs focus on liver function supporting, while ignore the immuno-inflammatory response caused by the inflammatory cytokines "second attack" [10]. Furthermore, several differentially expressed functional proteins within co-cultured hepatocytes have been revealed by comparative proteomics such as high mobility group box chromosomal protein 1 (HMGB1).

HMGB1 is a ubiquitously expressed DNA-binding protein that stabilizes nucleosome formation, facilitates gene transcription and regulates the activity of steroid hormone receptors [11]. Recently, HMGB1 has been established as a late mediator of lethal systemic inflammatory disease. By itself or in conjunction with other pro-inflammatory cytokines (e.g., IL-1β, IFN-γ and TNF-α), HMGB1 amplifies an inflammatory response by stimulating the release of various proinflammatory cytokines [12]. Meanwhile, RAGE was initially identified as a receptor for AGEs [13]. Since then, we have learned that this receptor has various binding partners. Rather than binding to a single specific ligand or even a group of closely related ligands, RAGE binds to several classes of molecules that lack sequence similarities. These ligands include HMGB1.

In light of the important role of HMGB1 in inflammatory in ALF and the exploration of the relationship between HMGB1 and RAGE, our primary goals were (a) to determine the plasma and hepatic tissue levels of HMGB1 in a drug-induced model of ALF in rats and (b) to confirm a protective effect of specific anti-HMGB1 antibodies and the HMGB1 antagonist nicotine in the ALF model.

Materials and methods

Animals

Male Sprague-Dawley rats, weighing approximately 200 to 250 g, were used for this study. All animals were acclimated to the animal research laboratory for 5 days before experiments and were maintained in a light-controlled room (12-h light/dark cycle) at an ambient temperature of 25°C with chow diet and water. All animal procedures were performed according to institutional and national guidelines and approved by the Animal Care Ethics Committee of Nanjing University and Nanjing Drum Tower Hospital.

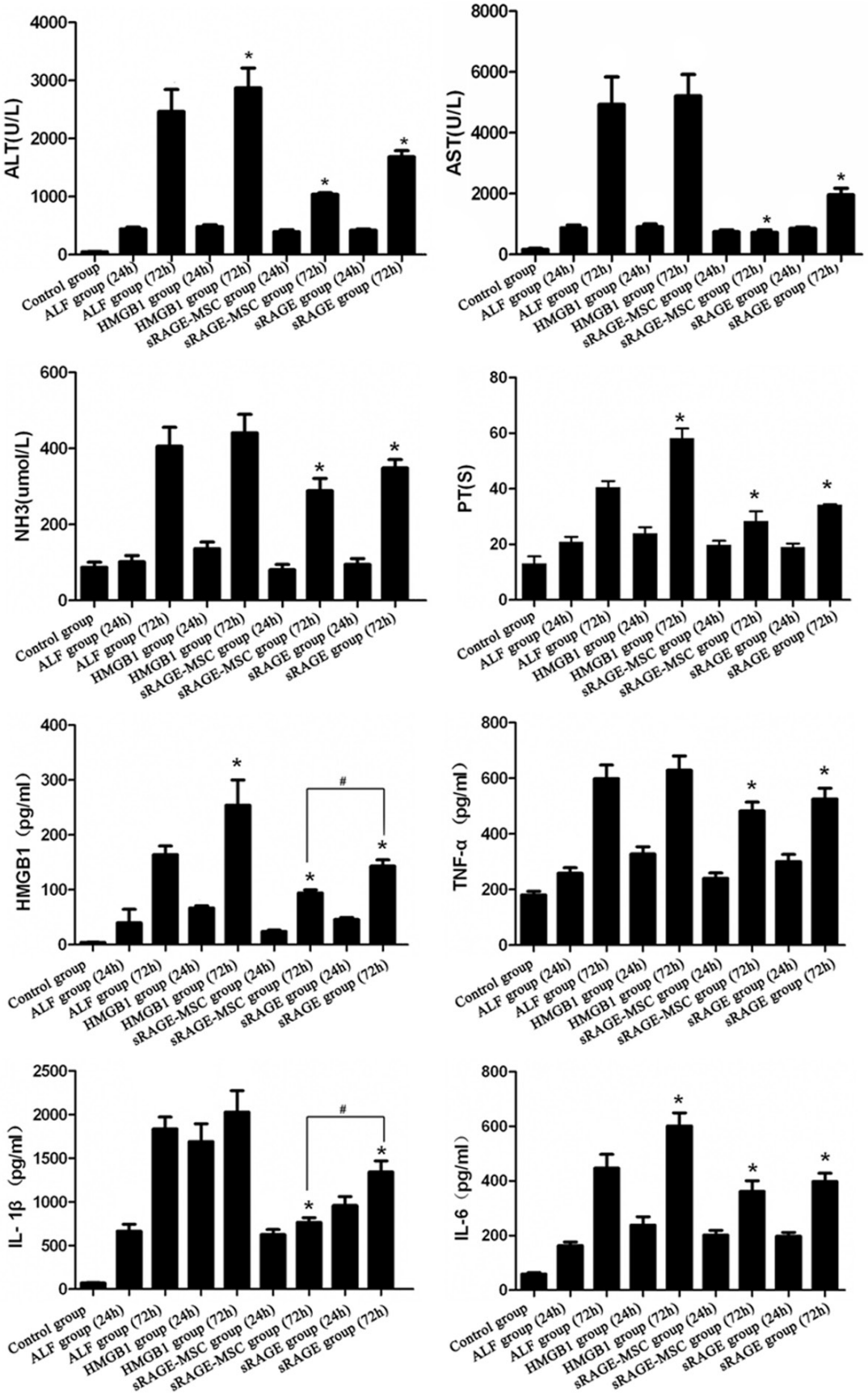
ALT(U/L)
AST(U/L)
NH3(umol/L)
PT(S)
HMGB1 (pg/ml)
TNF-α (pg/ml)
IL-1β (pg/ml)
IL-6 (pg/ml)
Control group
ALF group (24h)
ALF group (72h)
HMGB1 group (24h)
HMGB1 group (72h)
sRAGE-MSC group (24h)
sRAGE-MSC group (72h)
sRAGE group (24h)
sRAGE group (72h)

Figure 2. Plasma NH3, AST, ALT and PT of each groups were measured at 24 h and 72 h after the model was established. Compared to normal group, ALT, AST, PT and NH3 of model group significantly increased (12 h, $P<0.05$), and aggravated over time (72 h, $P<0.05$), the same tend can be observed in rHMGB1 group. Giving recombined-sRAGE and sRAGE-transfected MSCs reverse the aggravating tendency. ELISA was applied to determine the expression of HMGB1 and other cytokines in plasma collected at 24 h and 72 h. HMGB1 as well as TNF-α, IL-1β and IL-6 of each groups were measured and compared in histograms. Expression of HMGB1 as well as TNF-α, IL-1β and IL-6 are low in the plasma of normal group. The expressions were markedly raised after the model was established. For HMGB1 group at 24 h after administration, however, expression of HMGB1 is significantly higher than normal group, and the peak was reached at 72 h, this trend is also shown in TNF-α, IL-1β and IL-6, indicating severe inflammation due to ALF. The high expressions of indexes in HMGB1 group were markedly suppressed when recombinant-sRAGE or sRAGE-transfected MSCs was given. The suppression extend has no obviously difference for sRAGE group than for GFP/sRAGE-transfected MSCs group.

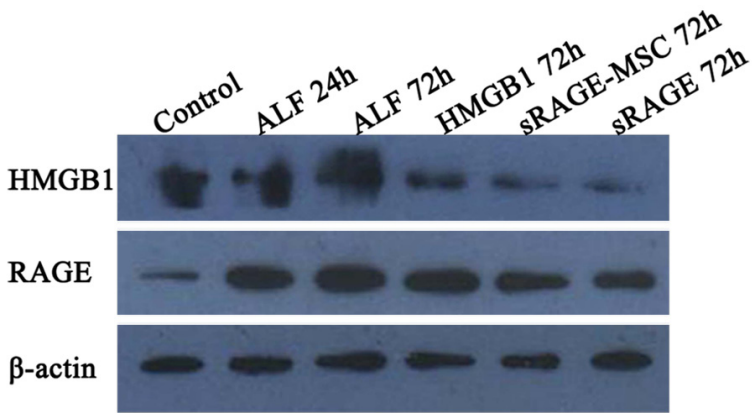

Figure 3. Western Blot was used to determine the expression of HMGB1 and RAGE in liver. MGB1 expression of model group in liver is distinctly higher at 12 h, even higher at 72 h, and RAGE expression is in accordance with HMGB1 expression. The RAGE expression of normal group at 72 h is higher than normal group, but at the same level of model group. Recombinant-sRAGE and sRAGE-transfected MSCs reduce the HMGB1 level.

Reagents

L-DMEM medium, fetal bovine serum and trypsin were purchased from Gibco (Grand Island, NY). For in vitro experiments, recombinant HMGB1 proteins were obtained from Abcam (Cambridge, MA). Purified sRAGE and Srage ELASA Kit were purchased from ADIPOBIOSCIENCE. RAGE was purchased from Abcam (Cambridge, MA). D-Gal was purchased from Sigma (St. Louis, MO). Rat HMGB1, IL-1, TNF-α, and IL-6 ELISA Kit were purchased from BD (Pharmingen, La Jolla, CA). TUNEL Kit was purchased from Thermo Fisher (Santa Cruz, CA).

Acute liver failure model

D-Gal was dissolved in 50 g/L glucose solution until a final concentration of 10% and pH was adjusted to 6.8. The animals were injected intraperitoneally with D-gal (0.6 g/kg) to induce Acute Liver Failure Model.

Cell culture

MSCs were purchased from Invitrogen and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin at 37°C with 5% CO_2.

Establishment of GFP/sRAGE-MSC

MSC line stably expressing rat GFP and sRAGE was constructed by using the lentivirus system according to the manufacturer's instructions, and cultured cells mentioned above. GFP/sRAGE-MSCs were cultured under routine conditions. Twenty days later, GFP expression was observed in up to 95.5% of the cultured MSCs and high fluorescence intensity was sustained, suggesting that GFP and sRAGE were successfully transduced into MSCs and could maintain a stable expression over a long time (**Figure 7**). Lentivirus infected in MSC cell line, the mRNA level of sRAGE genes has increased 6,000 times in lentivirus infected MSC cell line than in the control cell line (**Figure 8**).

Experimental groups and treatments

Male Sprague-Dawley rats were divided into five groups randomly. Control group was injected intraperitoneally with saline. ALF group was induced by d-galactosamine (0.6 g/kg) via intraperitoneal injection. HMGB1 group were treated with HMGB1 recombination protein (200 μg/kg) via penile vein right after ALF model induced. sRAGE group was injected

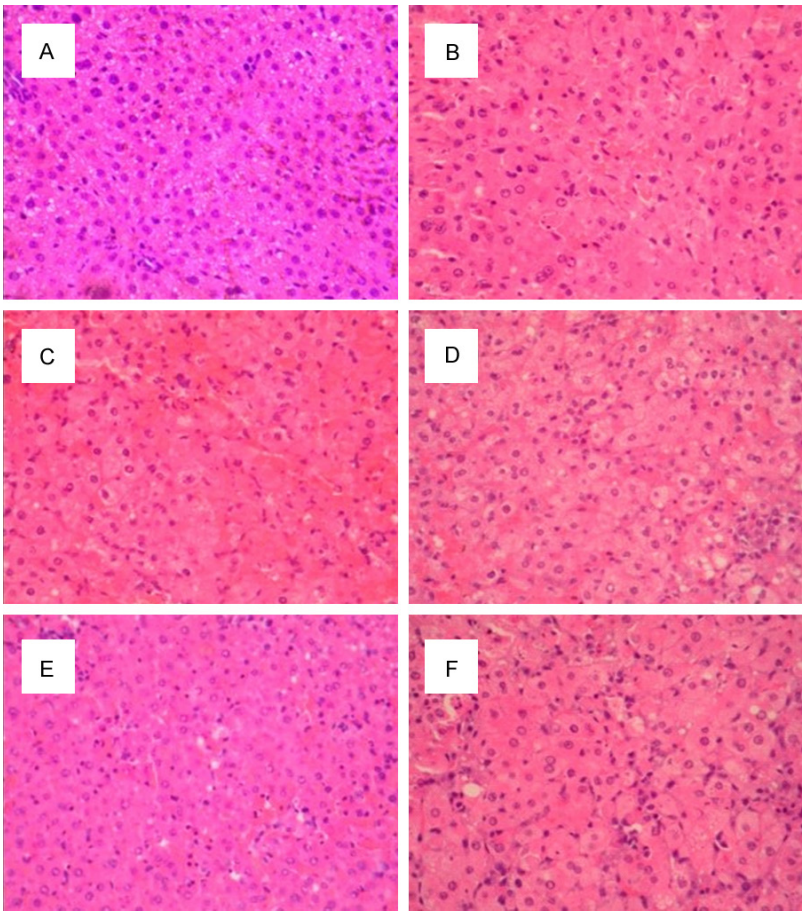

Figure 4. For HE staining, cytoplasm of liver tissue in control group was acidophilia. Central vein was surrounded by hepatocytes radiated out in all directions (A). 24 h (B) and 72 h (C) after the D-gal was challenged; apoptotic bodies and acidophilic change of hepatocytes can be observed, as well as infiltration of inflammation cell and spotty necrosis. In HMGB1 group, 2/3 of hepatocytes were observed necrosis and else phenomenon was in accordance with 72 h after modeling, but even severer (D). Administration of recombinant-Srage (E) or sRAGE-transfected MSCs (F) partly reversed these abnormalities in ALF group and HMGB1 group.

sRAGE recombination protein (400 μg/kg) via penile vein right after ALF model induced. sRAGE-MSC group received MSC transplantation (3×10^6) which could maintain a stable expression of sRAGE via penile vein right after ALF model induced. Liver function, level of cytokines and liver pathological changes were measured in the following days every 12 h. Overall survival was determined after 72 h for each group after the model was established.

Determination of plasma cytokines and HMGB1 level

Blood samples of each group were collected every 12 h after the model was established, centrifuged at 3000 rpm for 10 min at 4°C, and stored at -80°C. ELISA kits were used to quantify plasma levels of HMGB1, IL-1, TNF-α, and IL-6 according to the manufacturers' instructions.

Expression of HMGB1 and RAGE in liver

Liver tissues were collected at 72 h for each group. ALF group were collected at 12 h and 72 h. The levels of HMGB1 and RAGE in every 100 mg liver tissue were measured by Western blot. Tissues were homogenized in lysate buffer containing protease and phosphatase inhibitors. Equal amounts of proteins from each sample were resolved on 4% to 20% SDS-PAGE gels, transferred, and immunoblotted onto nitrocellulose membrane. Antibodies for HMGB1, RAGE and β-actin antibodies were used.

Distribution of HMGB1 and RAGE in Liver

Liver tissues were collected at 72 h for each group. ALF group was collected at 12 h and 72 h. The liver tissues were used for histological examination, and immunohistochemical staining was applied to determine HMGB1 and RAGE distribution. The samples embedded in paraffin were rehydrated and treated with 3% H_2O_2. The HMGB1 antibody or RAGE antibody was added and preserved at 4°C overnight. Biotin-labeled secondary antibody and streptavidin-biotin-peroxisome solution were added, and the samples were stained by DAB, restained by hematoxylin, and sealed with neutral gum. Samples treated with phosphate buffer served as the negative control.

Determination of liver cell apoptosis

The TUNEL assay kit was used to detect apoptosis. Paraffin sections were routinely deparaffinized, rehydrated, and then rinsed by PBS.

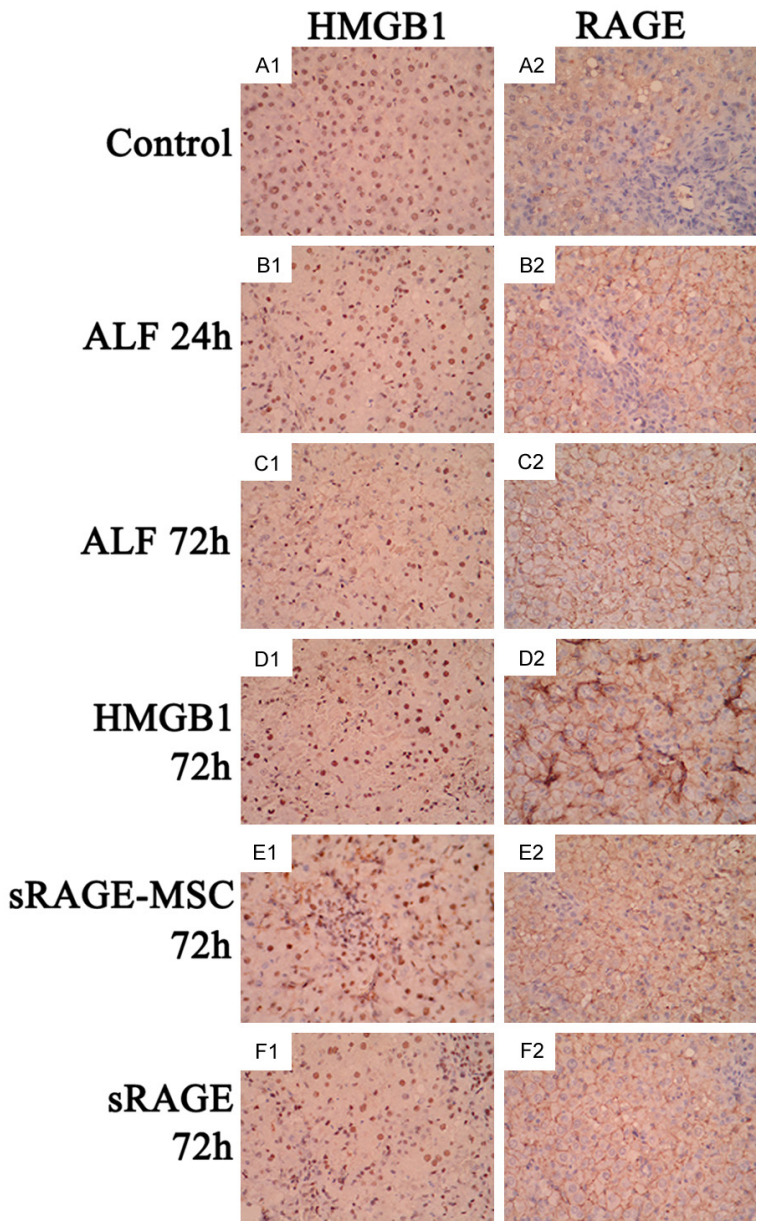

Figure 5. Immunohistochemical staining was applied to determine HMGB1 and RAGE distribution in liver tissue. Nuclei are lightly stained with brown, and hardly cytoplasm is stained in the control group (A). The number of HMGB1-positive cells in the nucleus increased compared to the control group at 24 h (B) and 72 h (C) after D-gal induction. After administration of HMGB1 (D), nuclei were distinctly dense stained, and cytoplasm is stained as well. sRAGE reversed the dense distribution of HMGB1 in nucleus (F). RAGE is cytomembrane localized. Net-shaped structure already emerged at 24 h after the model was established (B). RAGE expression was greatly enhanced by the administration of HMGB1 (D), hepatocytes was surrounded by thicker net-shaped structure even than ALF group at 72 h. The enhanced expression was reversed by giving recombinant-RAGE (F) and sRAGE-transfected MSCs (E).

After blocking endogenous peroxidase activity by methanol, permeability liquid (1 g/L TritonX-100 was dissolved in 0.1% sodium citrate), TUNEL reaction solution and Converter-POD were added. Each slice was stained by DAB, and liver cell apoptosis was observed under microscopy. The primary antibody was replaced by phosphate buffer on the positive specimens in negative control. Five fields were randomly selected in each slice under high-power field (400×). The number of positive liver cells and the total number of liver cells in each field were counted to calculate the percentage of positive liver cells.

Statistical analysis

All statistical analyses were performed with SPSS v.21.0. Data were reported as mean ± SD. Statistical significance was analyzed by Student t-test; $P<0.05$ was considered statistically significant.

Results

Survival rates

Survival of each group was recorded every 24 h, and the overall survival rate was determined at 72 h after treatment (**Figure 1**). For control group, ALF group, HMGB1 group, sRAGE-MSC group and sRAGE group, the overall survival rates were 100%, 30%, 10%, 60% and 50% respectively. According to the survival curve, survival rate of ALF model group significantly dropped over time than the control group, and the HMGB1 group presented a sharper drop than AFL model group. The dropping rate was much lower than sRAGE group, and similar as sRAGE-MSC group.

Liver function and cytokines level in plasma

The NH3, AST, ALT and PT in plasma of each group were measured at 24 h and 72 h after

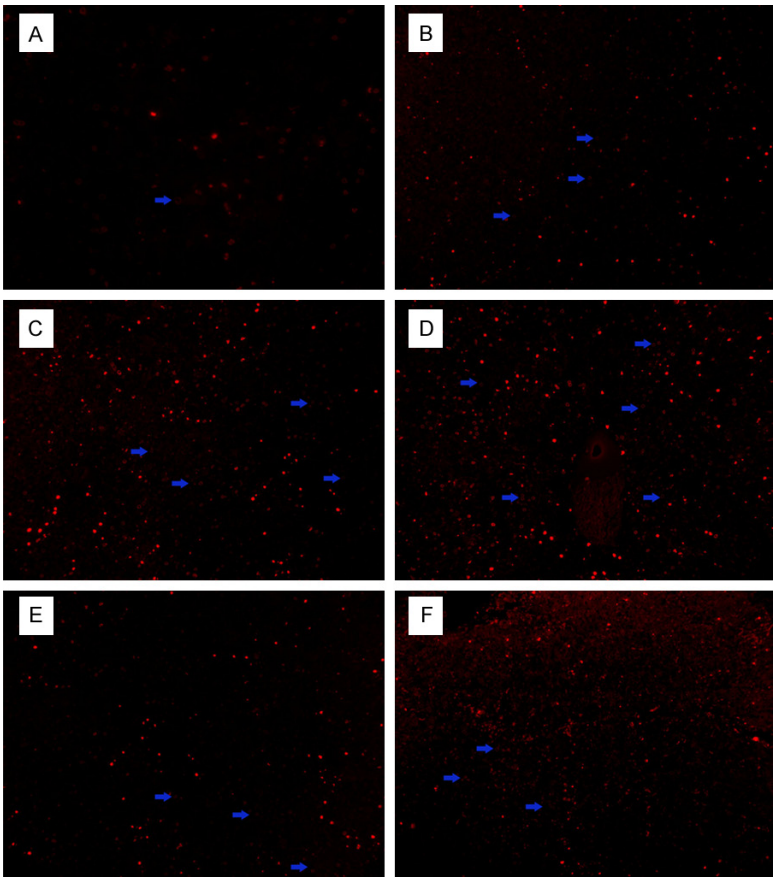

Figure 6. TUNEL assay was used to determine apoptosis level of hepatocytes of each group during ALF. There were hardly any apoptosis cells observed in the control group (A). The number of TUNEL stained cells was markedly increased in ALF group (B, C). The serious apoptosis level of HMGB1 group (D) was reversed to a relatively low state by giving recombinant-sRAGE (E), and the apoptosis level was closed to the normal state by sRAGE-transfected MSCs administration (F).

the model was established to determine liver function and compared in histograms (**Figure 2**). As shown in the figure, compared to control group (NH3 87.1±13.7g/L, AST 164.4±35.0 u/L, ALT 49.9±5.1 u/L, PT 13.1±2.6 s), ALT (440.1±32.9 u/L, 24 h), AST (871.7±94.2 u/L, 24 h) and NH3 (101.8±16.6 g/L) as well as PT (20.9±1.8 s, 24 h) of model group significantly increased (P<0.05), and aggravated over time (NH3 405.7±79.8 g/L, AST 4933.3±901.8 u/L, ALT 2466.6±378.5 u/L, PT 40.6±2.2 s, 72 h). The same tend can be observed in HMGB1 group, and each index is significantly higher than normal group (NH3 441.0±58.6 g/L, AST 5213.3±701.8 u/L, ALT 2870.3±344.9 u/L, PT 58.2±3.5, 72 h). The aggravating tendency was distinctly reversed by giving recombined-sRAGE (sRAGE group, NH3 288.5±32.9 g/L, AST 721.8±79.8 u/L, ALT 1039.2±25.3 u/L, PT 28.4±3.5, 72 h), as well as sRAGE-transfected MSCs (sRAGE-MSC group, NH3 348.6±22.1, AST 195-4.3±223.9 u/L, ALT 168-65.3±206.3 u/L, PT 34.2±0.3, 72 h). Reversing extent of sRAGE-MSC group is similar as sRAGE group, which is also reflected in survival rate. ELISA was applied to determine the expression of HMGB1 and other cytokines in plasma collected at 24 h and 72 h. HMGB1 as well as TNF-α, IL-1β and IL-6 of each group were measured. As shown in **Figure 2**, expression of HMGB1, TNF-α, IL-1β and IL-6 are low in the plasma of control group. However, after administrating the HMGB1 at 24 h, expression of HMGB1 was significantly higher than control group, and the peak reached at 72 h, this trend was also shown in TNF-α, IL-1β and IL-6. The high expressions of the above indexes in HMGB1 group were markedly suppressed when recombinant-sRAGE or sRAGE-transfected MSCs was given. The suppression extension has no obvious difference between sRAGE group and sRAGE-MSC group.

Expression of HMGB1 and RAGE in liver

Western blotting was performed to investigate the expression of HMGB1 and RAGE in liver tissues of each group. In the present study (**Figure 3**), HMGB1 expression in liver was significantly higher at 24 h compared with control group, and the increase was maintained to 72 h (P<0.05). HMGB1 expression in the HMGB1 group was also significantly higher than that in control group. However, sRAGE or sRAGE-MSC partially reversed the increasing (P<0.05). RAGE is one of the known receptors of HMGB1, as shown in the (**Figure 3**), the expression of RAGE was in accordance with HMGB1. In the ALF group, the RAGE expression at both 24 h

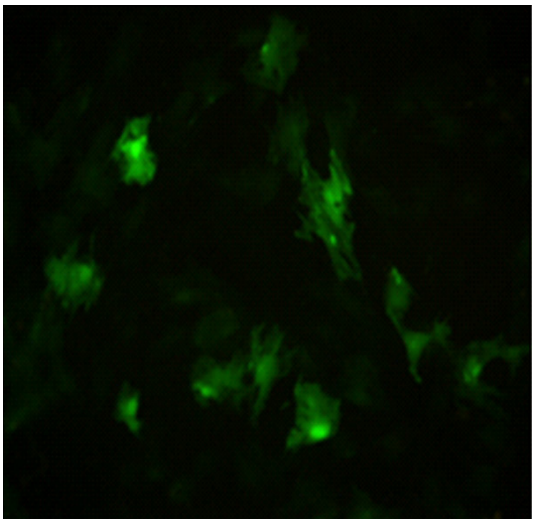

Figure 7. The level of GFP expressed in GFP/sRAGEMSC been observed by fluorescence microscope (400×). GFP/sRAGE-MSC was cultured under routine conditions. Twenty days later, GFP expression was observed in up to 95.5% of the cultured MSCs and high fluorescence intensity was sustained, suggesting that GFP and sRAGE were successfully transduced into MSCs and could maintain a stable expression over a long time.

and 72 h was significantly higher than control group, and was similar to the level of HMGB1 group. The administration of recombinant-sRAGE as well as sRAGE-MSC markedly reduced the RAGE expression compared with the HMGB1 group and the ALF group. However, there is no significant difference between the sRAGE and sRAGE-MSC group.

Effects of sRAGE-MSC on histopathological changes in D-gal challenged rats

According to **Figure 4**, cytoplasm of liver tissue in control group was acidophilia. Central vein surrounded by hepatocytes radiated out in all directions (**Figure 4A**). 24 h after the D-gal was challenged, apoptotic bodies and acidophilic change of hepatocytes can be observed, as well as infiltration of inflammation cell and spotty necrosis (**Figure 4B**). At 72 h, serious and extensive necrosis of hepatocytes occurred. More than 1/3 of hepatocytes were necrosis or bridging necrosis, disordered lobules of liver, fragmented hepatic cords, abnormally expanded bleeding sinus could be observed under microscopic examination, and a great amount of inflammation cells were infiltrated inside lobules, the other hepatocytes were distinctly steatosis (**Figure 4C**). In HMGB1 group, 2/3 of hepatocytes were observed necrosis and else phenomenon was in accordance with 72 h after modeling, but even severer (**Figure 4D**). Administration of recombinant-Srage (**Figure 4E**) or sRAGE-transfected MSCs (**Figure 4F**) partly reversed these abnormalities in ALF group and HMGB1 group, and no obvious differences were observed between these two groups.

Distribution of HMGB1 and RAGE in liver

Distribution of HMGB1 and RAGE were analyzed by immunohistochemical staining in the liver slices. HMGB1 was generally nucleus localized, and translocate to cytoplasm in inflammation, stress reaction and some other particular situation. D-gal and HMGB1 injection caused massive expression of HMGB1 in the liver. As shown in the **Figure 5**, nuclei were lightly stained with brown, and hardly cytoplasm was stained in the control group (**Figure 5A**). The number of HMGB1-positive cells in the nucleus increased compared with the control group at 24 h after D-gal induction (**Figure 5B**), and at 72 h nearly all nuclei were densely stained, the shape of nucleus was abnormal (**Figure 5C**). Cytoplasm of cell in model group was hardly stained, which means the translocation of HMGB1 is not obvious. However, after administration of HMGB1 (**Figure 5D**), nuclei were distinctly densely stained, and cytoplasm was stained as well, which means the existence of HMGB1 translocation from nucleus to cytoplasm. sRAGE reversed the dense distribution of HMGB1 in nucleus (**Figure 5F**) compared with HMGB1 group, and most nuclei were in their normal shape; Administration of sRAGE-transfected MSCs (**Figure 5E**) was in accordance with the effect of sRAGE group, and almost the same effect according to observation.

RAGE was cytomembrane localized. As a receptor of HMGB1, amount of RAGE and HMGB1 was closely related. RAGE was represented by the brown stain on the edge of hepatocytes in **Figure 5**, and overexpression of RAGE appeared to be net-shaped structures surrounding cells. In the figure of control group (**Figure 5A**), due to the normal expression of RAGE, distribution of RAGE on the cytomembrane was uniform and sparse. Net-shaped structure already emerged at 24h after the model was established (**Figure 5B**), and developed until distinctly existing

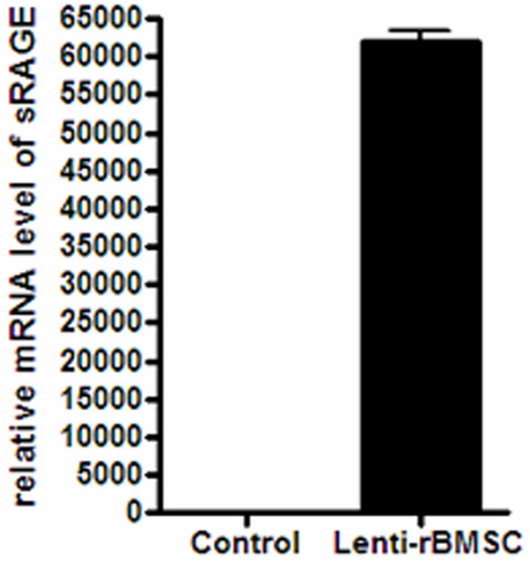

Figure 8. The mRNA level of sRAGE genes expressed in GFP/sRAGE-MSC. Lentivirus infected in MSC cell line, the mRNA level of sRAGE genes has increased 6,000 times in lentivirus infected MSC cell line than in the control cell line.

at 72 h (**Figure 5C**), which indicated the increasing expression of RAGE over time. RAGE expression was greatly enhanced by the administration of HMGB1 (**Figure 5D**), hepatocytes were surrounded by even thicker net-shaped structure than ALF group at 72 h. The enhanced expression was reversed by given recombinant-RAGE (**Figure 5F**) and sRAGE-transfected MSCs (**Figure 5E**). Net-shaped structure reduced on both treatments and the sharp edges of cells reemerged. Overall, the change of RAGE has consistent tendency of HMGB1 due to their close relation.

Determination of hepatocytes apoptosis

Apoptosis is a direct result of ALF, and TUNEL assay was used to determine the apoptosis level of hepatocytes of each group during ALF. As shown in the **Figure 6**, solid red spots represented non-specific erythrocyte stain, and the hollow circles marked by arrows were stained apoptosis nuclei. The result showed that there were hardly any apoptosis cells observed in the control group (**Figure 6A**). The number of TUNEL stained cells markedly increased in ALF group (**Figure 6B**), demonstrating that apoptosis greatly occurred in the D-gal challenged liver. The HMGB1 group, however, showed even more TUNEL positive cells than ALF group in the **Figure 6D**, suggesting that apoptosis is a result of HMGB1 mediated ALF. The serious apoptosis level of HMGB1 group was reversed to a relatively low state by giving recombinant-sRAGE (**Figure 6E**), and the apoptosis level was closed to the normal state by sRAGE-transfected MSCs administration (**Figure 6F**). The protective effect of sRAGE on inhibiting apoptosis confirmed the effect of HMGB1 in ALF development.

Discussion

ALF has been currently recognized as a global problem to all clinicians. BAL is a safe and effective therapeutic modality for patients with end-stage liver diseases. It will serve as a bridge to liver transplantation or regeneration, which is needed to reduce the morbidity and mortality caused by ALF. Furthermore, several differentially expressed functional proteins within co-cultured hepatocytes in BAL have been revealed by comparative proteomics such as HMGB1. So the inevitable trend for BAL nowadays is to suppress systemic immuno-inflammatory reaction provoking the conversion of BAL from passive support to active intervention.

Since the first report of HMGB1 as “death mediator” in 1999 [11] a growing number of articles have reported that HMGB1 plays a fundamental role in various disease conditions, including acute pancreatitis [14], ARDS [15], sepsis [16] and other diseases [17-21]. In this study, we determined not only the plasma levels, but also the hepatic tissue levels of HMGB1 in a rat model of ALF induced by D-galactosamin and further investigated the protective effect of sRAGE.

Compared with the control group, the levels of HMGB1, IL-1, IL-6 and TNF-α in ALF group and HMGB1 group rapidly increased after ALF induction and showed a peak at 72 h [22] (**Figure 2**). The similar trend can be observed in monitoring indexes like liver function, blood clotting and blood ammonia (**Figure 2**). Our finding suggests that the plasma levels of HMGB1 is an important indicator for ALF severity [23]. As compared with the control group, the levels of HMGB1 in homogenized hepatic tissue supernatants increased obviously after ALF induced detected by WB and IHC. A large number of animal experiments and clinical studies

demonstrated that specifically inhibited of HMGB1-mediated inflammatory response can significantly improve the severe acute pancreatitis, sepsis, septic shock and ARDS. It can be considered as an important indicator for inflammatory diseases [24]. Different from some classic type therapy targets for immuno-inflammatory diseases, HMGB1 has been defined as an advanced stage mediator of inflammation with a wide therapeutic interval. Even in the advanced phase of clinical pathogenesis of inflammation, patients can still benefit from the anti-HMGB1 therapy [13]. Therefore, HMGB1 is considered to be a promising intervention target in inflammatory diseases. It is undoubtedly an important effective molecule and plays an important role in many pathological, as well as in chronic inflammatory diseases.

HMGB1, the extracellular mediator of inflammation, only combined with the membrane receptors outside the immune cells, can make the biological effects operate smoothly. Rather than binding to a single specific ligand or even a group of closely related ligands, RAGE [25, 26] has various binding partners. These ligands include HMGB1, and it has natural high affinity with HMGB [27]. It is widespread but relatively low expression of RAGE on vascular endothelial cells, neutrophils, monocytes/macrophages, lymphocytes, DCs, cardiomyocytes, and neurons [28]. Our finding suggests that RAGE expression increases in hepatic tissue levels when inflammatory mediators such as HMGB1 accumulate in the D-galactosamine-induced ALF model. But, it maintains at a relatively low level in Srage group and sRAGE-MSC group (**Figure 3**). Reportedly, RAGE promoter contains multiple functional NF-κB and SP-1 transcription factor-binding sites [29, 30], ligands and proinflammatory cytokines can promote the expression of RAGE [31], potentially triggering a receptor-dependent autoinflammatory loop [32].

Recently, soluble RAGE (sRAGE), one of RAGE variants has been indentified in human and mouse lung. It is the extracellular (soluble) domain of RAGE at the V-, C1- and/or C2-immunoglobulin like domains [33]. Due to lack of the intracellular region, sRAGE can not transmit biology signal into intracellular. Therefore, it can competitively bind to HMGB-1 with RAGE on cell surface. Experiments using sRAGE as a ligand decoy have illustrated largely beneficial effects in reducing inflammatory stress, thereby, preventing long-term tissue damage in models of diabetes and immune/inflammatory disorders [34-37]. The present project aims to take bone marrow mesenchymal stem cells (MSC) as a vehicle for sRAGE gene so as to consistently express proteins which could competitively bind to HMGB-1 with RAGE within liver, thus would suppress subsequent immuno-inflammatory reaction. Our present study demonstrated that administrating sRAGE recombinant protein to Srage group or transfecting sRAGE gene to sRAGE-MSC group after the ALF model was established may significantly prolong ALF rat survival time (**Figure 1**), as well as improve its liver functions (**Figure 2**), suppressed the inflammatory cytokines secretion (**Figure 2**), alleviated hepatocytes swelling and necrosis (**Figure 6**), which indicate measurement of sRAGE levels in vivo holds promise for the identification of potential therapeutic targets and/or biomarkers of RAGE activity in disease. But sRAGE is a kind of small molecule protein with some disadvantages, such as a short circulating half-life, needs to be repeatedly administrated, and cost a lot [35]. The sRAGE-MSC group is capability to overcome the disadvantages of the sRAGE group mentioned above. MSCs have been considered as a mature gene expression platform while hepatocytes in BAL can support the liver function. We have successfully established the MSC co-cultured with hepatocytes as a vehicle for sRAGE gene so as to consistently express proteins which could competitively bind to HMGB-1 with RAGE on cell surface within liver, thus would suppress subsequent immuno-inflammatory reaction.

Although the underlying mechanisms of sRAGE used as HMGB1 blockade in the ALF model remain to be systematically investigated. It warrants the inevitable trend for BAL development nowadays is to suppress immuno-inflammatory response, which provokes the conversion of BAL from passive support to active intervention. Recently emerging concept in HMGB1 biology is its ability to interact with other molecules, including DNA, lipopolysaccharide (LPS), and also with nucleosomes that augment or modify the function of HMGB1 itself. If HMGB1 is associated with DNA, then the complex signals through RAGE/TLR9. A major question in this

field was how RAGE transduces signals from the cell surface to the nucleus. Several papers have tried to address this question by searching for direct binding partners, using the cytoplasmic tail of RAGE as bait. Ishihara et al. [38] identified both extracellular signal-related kinase-1 and -2 (ERK1/2) as direct RAGE-binding partners. Further truncation of the cytoplasmic domain unveiled a putative ERK docking site at the membrane-proximal region. HMGB1 stimulation of RAGE-transfected HT-1080 cells induced the interaction of RAGE and ERK1/2. Hudson et al. [31] employed a yeast two-hybrid system to explore possible cytoplasmic-binding partners of RAGE. Results from this study indicate that the FH1 domain of mammalian Diaphanous-1 (mDia-1) interacts with RAGE. If HMGB1 is associated with the nucleosome, signaling is strictly TLR2 dependent. HMGB1 also has the potential to bind to LPS [39]. HMGB1, like LPS-binding protein, can actively destabilize LPS aggregates and present LPS monomers to CD14, thus increasing the overall sensitivity of the TLR4/MD-2 receptor complex.

Although the focus of this study is the role of HMGB1 and the receptor RAGE in ALF, HMGB1-TLR4 may also be important and needs to be investigated in the future. We will explore in depth the enrichment and development in modern idea of ALF combined treatment. If successful, the present project will provide a molecular target for configuration of a brand-new BAL system with dual functions of supporting liver functions and intervening immuno-inflammatory reaction. The expected findings from this study will provide theoretical foundation and beneficial exploration for the application of the concept of suppressing immuno-inflammatory reaction to the comprehensive treatment of ALF.

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Disclosure of conflict of interest

None.

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