

## Original Article

# Lowering blood ammonia prevents hepatocyte injury and apoptosis

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Received April 7, 2015; Accepted July 2, 2015; Epub August 15, 2015; Published August 30, 2015

**Abstract:** To study hepatocyte injury through establishing the rat model of acute hepatic failure (ALF). ALF rat model was established by administration with D-galactosamine and LPS, and then giving lowering blood ammonia (LBA) treatment. Besides, the intervention groups were injected with ornithine and aspartate. The control groups were injected saline. Blood ammonia, ALT, AST, TNF- $\alpha$  and IL-6 in blood samples were test at 12 hrs and 24 hrs after treatment with LBA. Hepatocyte apoptosis were tested by TUNEL and DNA Ladder. Expression of P53 and SPP1 were detected by RT-PCR. Results: showed that blood ammonia in hepatic failure group and intervention group compared with blank control group was significantly increased at 12 h, 24 h; intervention group compared with hepatic failure group was significantly reduced ( $P<0.05$ ). Serum ALT, AST in 24 h group were higher than 12 h. 12 h intervention group was decreased compared with hepatic failure group, but there was no significant statistically difference ( $P>0.05$ ). 24 h intervention group compared with hepatic failure group was significantly reduced ( $P<0.05$ ). Except the control group, DNA ladder and the TUNEL results showed hepatocyte apoptosis rate increased in 24 h compared with 12 h. Intervention group compared with hepatic failure group was significantly reducing ( $P<0.05$ ). IL-6, TNF- $\alpha$ , p53 expression levels were increased with time (24 h>12 h). The hepatic failure and intervention group compared with blank control group was significantly increased; Intervention group compared with hepatic failure group was significantly reducing ( $P<0.05$ ). SPP1 gene was high expression in ALF rat model. SPP1 level in hepatic failure and intervention group compared with control group was significantly increased, and intervention group compared with hepatic failure group was significantly reducing ( $P<0.05$ ). In conclusion, hepatocyte apoptosis is an important pathological change in ALF rat mode, and lowering ammonia can reduce liver injury and apoptosis. Blood TNF- $\alpha$ , IL-6 and SPP1 may be more sensitive injury indicators.

**Keywords:** Acute liver failure, blood ammonia, apoptosis

## Introduction

Liver failure (hepatic failure, HF) is a life-threatening critical illness requiring intensive care due to various causes [1, 2]. The patient will show varying severity of encephalopathy, hepatorenal syndrome, bleeding. The mortality rate as high as 70% to 80% [3, 4]. Elevated blood ammonia is one of the important causes of a series of abnormal performance [5, 6]. High ammonia can cause abnormal energy metabolism, oxidative stress, high mitochondrial permeability, gene transcription disorder, abnormal GABA and other neurotransmitter systems in brain, resulting in astrocytes disorder and

cerebral edema [7, 8]. However, high ammonia toxicity to different cells are not equal, such as: ammonia concentration does not inhibit Chinese hamster ovary cell line (CHO) growth [9, 10]. Millimolar concentrations of ammonia slightly inhibit the growth of pituitary GH (4) cells, inhibit the growth of kidney tissue LLC-PK (1) cells, but does not cause cell death; ammonia accumulation can cause the death of lymphoid tissue Jurkat cells in S phase [11, 12]. The rat bone marrow mesenchymal stem cells (MHC) are more sensitive to ammonia-induced growth inhibition than human MSCs when subjected to ammonia [13].

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**Table 1.** Ammonia value of the test results

Groups	Ammonia values ( $\mu\text{mol/L}$ )	
	Mean $\pm$ standard deviation	deviation of 95% confidence interval
12 h control group	33.13 $\pm$ 3.980	29.80~36.45
12 h liver failure group	199.38 $\pm$ 48.205	159.07~239.68
12 h intervention group	121.50 $\pm$ 14.755 <sup>a</sup>	109.16~133.84
24 h control group	33.75 $\pm$ 3.196	31.08~36.42
24 h liver failure group	154.00 $\pm$ 20.894	136.53~171.47
24 h intervention group	95.25 $\pm$ 7.106 <sup>b</sup>	89.31~101.19

In intervention groups and liver failure groups, Ammonia value were higher at 12 h than those at 24 h, and were significantly higher than those in the control group. Intervention group was significantly lower compared with liver failure group (a: 12 h Intervention group vs 12 h liver failure group,  $P < 0.05$ ; b: 24 h Intervention group vs 24 h liver failure group,  $P < 0.05$ ).

We found that ammonia lowering drugs (ornithine aspartate) significant recover liver injury indicators in patients with liver failure, improve clinical symptoms, and reduce the mortality rate [14]. The level of ammonia in patients with acute liver failure are closely related with mortality directly [15]. Therefore, accumulating the process of elevated blood ammonia in the liver cells may affect the liver again. We established an animal model of chronic hyperammonemia and found indicators of liver injury (ALT, AST) were significantly elevated. The reason of liver cell damage in rat model induced by ammonia is apoptosis, not inflammation, necrosis or edema [16]. So hyperammonemia may promote apoptosis occurred in the liver cells. Through establishing the rat model of ALF and then giving lowering ammonia drugs for treatment in early stage, the study focuses on the influence of early lowering blood ammonia on hepatocyte apoptosis and explore the possible mechanism of liver damage caused by hyperammonemia.

## Materials and methods

### Experimental animals

Forty-eight female SD rats, weighing 190-230 g, were obtained from Henan Experimental Animal Center [SCXK (Henan) 2005-0001]. The rats were maintained in the laboratory for animal experimentation in a specific pathogen-free environment, in laminar air-flow conditions with a 12-hour light-dark cycle, and at a temperature of 22°C to 25°C. All animals had free access to standard laboratory mouse food and water. All procedures were approved by the

Committee of Ethics in Animal Experiments at Zhengzhou University, and all animals were provided humane care in compliance with the institutional guidelines of Zhengzhou University.

### Animal grouping and treatment

The rats were randomly assigned to three groups: The control group (n=16) received normal saline (5 ml/kg) by intraperitoneal injection; Liver failure model group (n=16) were given LPS (100  $\mu\text{g/kg}$ ) and D-Gal (450 mg/kg) by intraperitoneal injection; The intervention group given LPS (100  $\mu\text{g/kg}$ ), D-Gal (450 mg/kg) and the addition of ornithine aspartate (1.5 g/kg) intraperitoneal injection. And then repeat intraperitoneal injection with ornithine aspartate or saline at 6 h, 12 h and 18 h, respectively. Twelve and twenty-four h after treatment, animals were sacrificed.

### Detection of ammonia, ALT, AST, IL-6, TNF- $\alpha$ by ELISA

After anesthetizing with pentobarbital, 3 ml cardiac blood samples were collected from rats, 1 ml of them was put into lithium heparin anticoagulant blood collection tube and placed immediately in cryopreservation ice box, send to the emergency laboratory in the First Affiliated Hospital of Zhengzhou University within 15 min. Ammonia were detected by "dry chemical direct chromogenic assay". The other 2 ml blood samples were placed in ordinary tubes and put in 37°C incubator for 30 min, then centrifuge 3000 rpm for 10 min. Supernatant were extracted and saved in -20°C. ALT, AST, IL-6 and TNF- $\alpha$  level were detected by ELISA.

### HE staining

Liver tissue were fixed by 4% paraformaldehyde, dehydrated, embedded in paraffin, HE staining changes were double-blind read by two Pathology physicians (Department of Pathology in the First Affiliated Hospital of Zhengzhou University) under an optical microscope photograph.

### TUNEL analysis

The staining of apoptotic cells was according to Roche TUNEL kit instructions. At high optical

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**Table 2.** Levels of ALT and AST in the rat serum

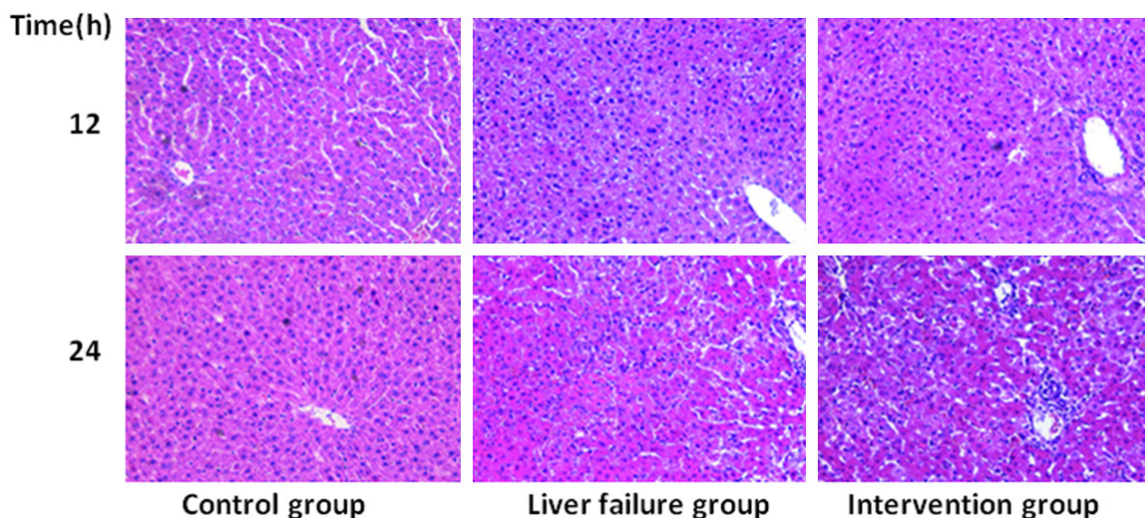
Groups	ALT (U/L)		AST (U/L)	
	Mean ± standard	deviation of 95% confidence interval	Mean ± standard	deviation of 95% confidence interval
12 h control group	46.851±4.884	42.335~51.368	150.481±9.502	141.694~159.270
12 h liver failure group	67.859±3.750	64.724~70.994	238.270±13.299	227.152~249.388
12 h intervention group	65.010±4.756 <sup>a</sup>	61.034~68.986	209.811±12.010 <sup>a</sup>	189.771~232.852
24 h control group	47.633±3.693	44.545~50.720	149.438±4.579	145.609~153.266
24 h liver failure group	80.061±4.969	75.907~84.216	279.598±12.820	268.881~290.316
24 h intervention group	70.851±4.787 <sup>b</sup>	66.849~74.853	249.591±13.495 <sup>b</sup>	238.309~260.874

ALT, AST significantly increased except for the control group. ALT and AST values were higher in liver failure group and intervention group compared to the control group. ALT and AST values were reduced in the intervention group compared with the liver failure group at 12 h but had no significant difference (a: 12 h Intervention group vs 12 h liver failure group,  $P>0.05$ ), but they were decreased significantly at 24 h (b: 24 h Intervention group vs 24 h liver failure group,  $P<0.05$ ).

**Table 3.** Serum IL-6 and TNF- $\alpha$  were detected by ELISA

Groups	IL-6 (pg/ml)		TNF- $\alpha$ (pg/ml)	
	Mean ± standard	deviation of 95% confidence interval	Mean ± standard	deviation of 95% confidence interval
12 h control group	42.424±1.179	41.439~43.410	82.351±9.906	74.070~90.632
12 h liver failure group	81.361±1.354	80.229~82.493	125.892±11.309	116.438~135.346
12 h intervention group	60.462±1.103 <sup>a</sup>	59.539~61.384	104.635±5.872	99.72~109.543
24 h control group	42.902±1.453	41.68~44.117	84.709±9.299	76.93~92.483
24 h liver failure group	92.299±1.771	90.818~93.780	159.331±13.457	148.081~170.581
24 h intervention group	65.126±2.402 <sup>b</sup>	63.11~67.133	116.078±13.398	104.876~127.279

Serum IL-6 and TNF- $\alpha$  expression levels were significantly increased in liver failure group and intervention group than the control group. IL-6 and TNF- $\alpha$  values were reduced in the intervention group compared with the liver failure group at 12 h and 24 h (a: 12 h Intervention group vs 12 h liver failure group,  $P<0.05$ ; b: 24 h Intervention group vs 24 h liver failure group,  $P<0.05$ ).

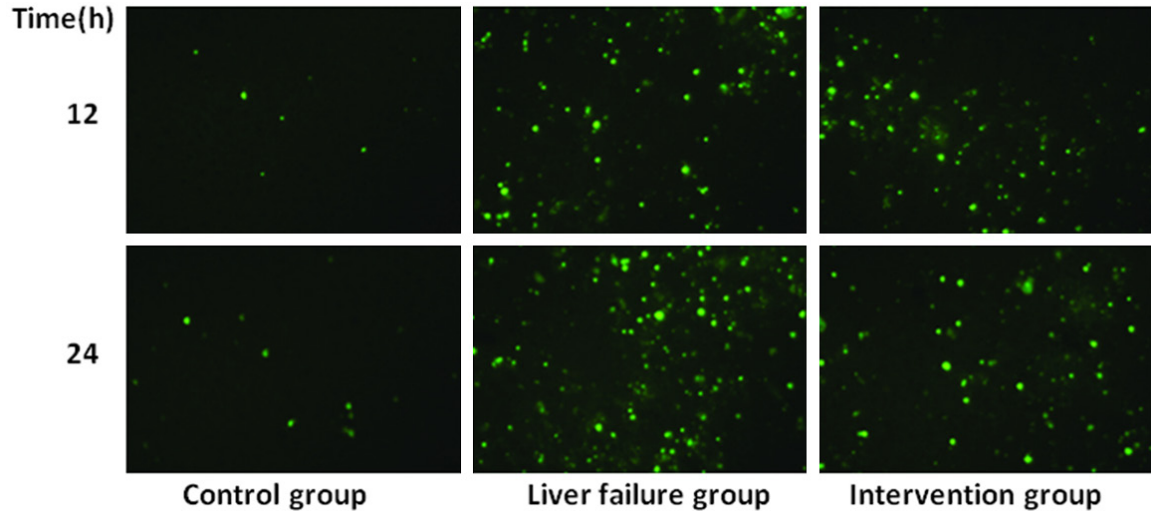


**Figure 1.** Liver HE staining pathological results after 12 h and 24 h treatment. Control group received normal saline (5 ml/kg) by intraperitoneal injection; Liver failure group were given LPS (100 ug/kg) and D-Gal (450 mg/kg) by intraperitoneal injection; Intervention group given LPS (100 ug/kg), D-Gal (450 mg/kg) and the addition of ornithine aspartate (1.5 g/kg) intraperitoneal injection.

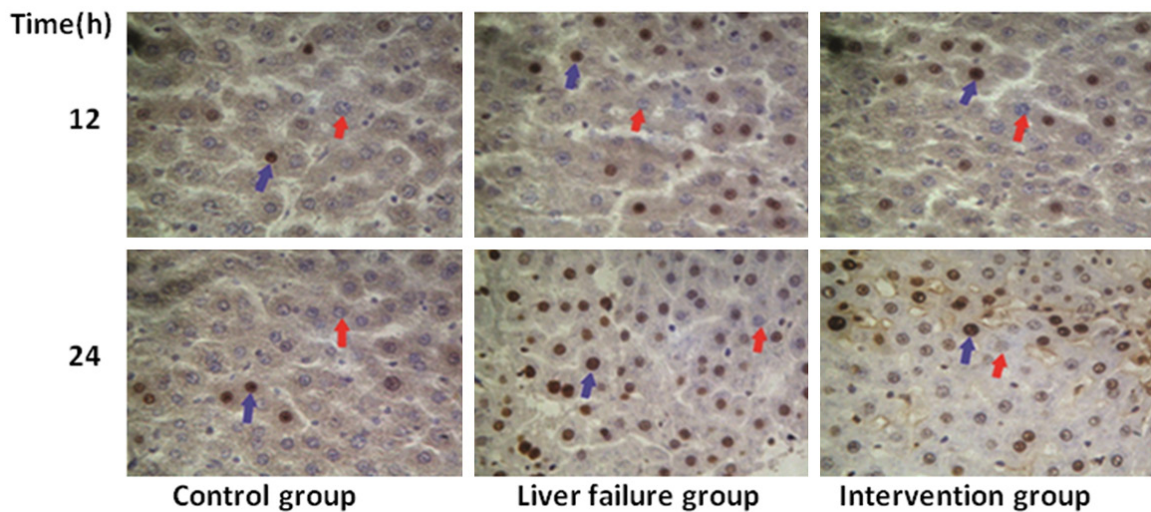
microscopy ( $\times 400$ ), five slides were observed in each group. Each slide randomly selected 10

visions. Apoptotic index (AI) = number of apoptotic cells/total cells  $\times 100\%$ .

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**Figure 2.** Apoptosis cells after 12 h and 24 h treatment were observed under fluorescence microscope (TUNEL  $\times 200$ ). Fluorescently labeled apoptosis increased in liver failure group and intervention group compared with the control group.



**Figure 3.** Apoptosis cells observed under Optical microscopy (TUNEL  $\times 400$ ). The dark brown apoptotic cells Stained by diaminobenzidine increased in liver failure group and intervention group compared with the control group.

**Table 4.** Results of apoptosis index

Groups	Apoptosis rate (%)	
	Mean $\pm$ standard	deviation of 95% confidence interval
12 h control group	5.500 $\pm$ 2.976	3.33~4.17
12 h liver failure group	43.875 $\pm$ 3.523	40.93~46.82
12 h intervention group	27.625 $\pm$ 4.926 <sup>a</sup>	23.51~.31.74
24 h control group	5.625 $\pm$ 2.722	3.35~7.90
24 h liver failure group	65.250 $\pm$ 4.713	61.31~69.19
24 h intervention group	37.500 $\pm$ 5.014 <sup>b</sup>	33.31~41.69

Except for the control group the apoptosis in 24 h were higher than 12 h (a: 12 h Intervention group vs 12 h liver failure group,  $P < 0.05$ ; b: 24 h Intervention group vs 24 h liver failure group,  $P < 0.05$ ).

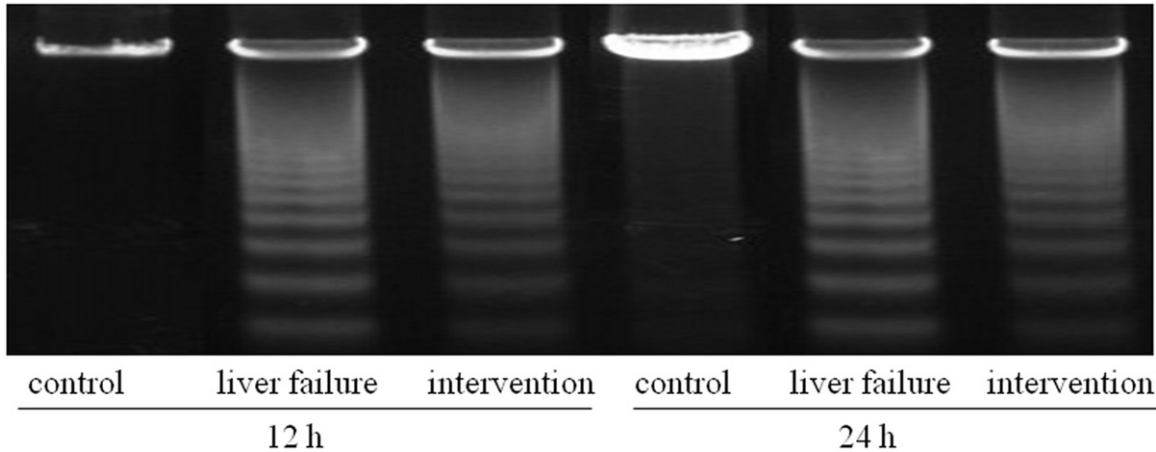
### DNA ladder

According to Beijing Ding Guo Biotechnology Co. Ltd. kit instructions, DNA were extracted and detected by UV spectrophotometer to observe whether there is DNA Ladder.

### RT-PCR

Total RNA was diluted to 500 ng/L and stored in  $-80^{\circ}\text{C}$ . The primers of P53 and SPP1 were design by Primer 5.0 software and synthesized by Sangon Biological

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**Figure 4.** Electrophoresis of DNA Ladder.

**Table 5.** The relative expression of P53 gene

Grouping	The relative expression of P53 gene	
	Mean $\pm$ standard deviation	deviation of 95% confidence interval
12 h control group	0.5219 $\pm$ 0.0121	0.5119~0.5320
12 h liver failure group	0.9417 $\pm$ 0.0122	0.9315~0.9519
12 h intervention group	0.8232 $\pm$ 0.0185 <sup>a</sup>	0.8077~0.8386
24 h control group	0.5286 $\pm$ 0.0116	0.5088~0.5283
24 h liver failure group	0.9704 $\pm$ 0.0132	0.9594~0.9814
24 h intervention group	0.8301 $\pm$ 0.0159 <sup>b</sup>	0.8167~0.8434

P53 relative expression levels were increased in liver failure group and intervention group compared with the control group. Intervention group decreased compared with liver failure group. Except for the control group the apoptosis in 24 h were higher than 12 h (a: 12 h Intervention group vs 12 h liver failure group,  $P < 0.05$ ; b: 24 h Intervention group vs 24 h liver failure group,  $P < 0.05$ ).

Engineering Co. The specific primer sequences are as follows: P53 forward 5'-CCAGGA-TGTTGCAGAGTTGTTA-3' and reverse 5'-CTCAC-GACCTCAGTCATGTGTT-3'; SPP1 forward 5'-CAT-TACAGCAAACACTCAGATGC-3' and reverse 5'-C-ATVAGAAAACAGGGAAACTCCT-3'. Expression data was normalized to the geometric mean of  $\beta$ -actin to control the variability in expression levels (forward primer 5'-TCAGGTCATCACTA-TCGGCAAT-3'; reverse primer 3'-AGAGGCAGG-GATGATGTTCTG-5') and calculated as  $2^{-[(Ct \text{ of P53 and SPP1}) - (Ct \text{ of } \beta\text{-actin})]}$ .

### Statistical analysis

Statistical analyses were performed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). The data are expressed as the means  $\pm$  SD. Student's t test was used to evaluate the

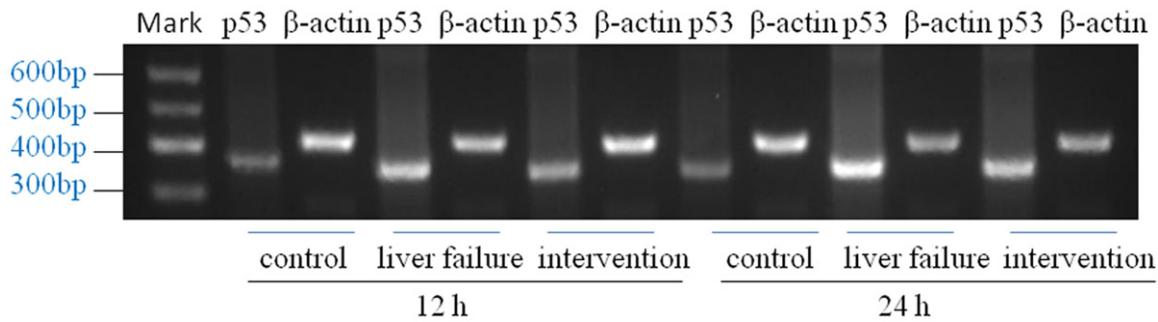
significance of the differences between two groups of data in all the pertinent experiments. A value of  $P < 0.05$  was considered statistically significant.

### Results

#### Blood sample test results and correlation analysis

In intervention groups and liver failure groups, level of ammonia were higher at 12 h than those at 24 h, and were significantly higher than those in the control group. Intervention group was significantly lower compared with liver failure group ( $P < 0.05$ , **Table 1**). Liver enzymes related indicators (ALT, AST) significantly increased except for the control group. ALT and AST values were higher in liver failure group and intervention group compared to the control group. ALT and AST values were reduced in the intervention group compared with the liver failure group at 12 h but had no significant difference ( $P > 0.05$ ), but they were decreased significantly at 24 h ( $P < 0.05$ ) (**Table 2**). Serum IL-6 and TNF- $\alpha$  expression levels were significantly increased in liver failure group and intervention group than the control group. IL-6 and TNF- $\alpha$  values were reduced in the intervention group compared with the liver failure group at 12 h and 24 h ( $P < 0.05$ ) (**Table 3**). ALT, AST and Ammonia Pearson correlation analysis ( $R_{ALT} = 0.785$ ,  $R_{AST} = 0.785$ ,  $P < 0.01$ , two-tailed test) showed positive correlation. IL-6, TNF- $\alpha$  and ammonia Pearson correlation analy-

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**Figure 5.** Electrophoresis of P53 gene amplification product. Intervention group decreased compared with liver failure group. Except for the control group the apoptosis in 24 h were higher than 12 h.

**Table 6.** The relative expression of SPP1 gene

Grouping	SPP1 gene	
	Mean ± standard deviation of 95% confidence interval	
12 h control group	0.5947±0.0156	0.5817~0.6077
12 h liver failure group	0.9572±0.0271 <sup>a</sup>	0.9345~0.9798
12 h intervention group	0.9392±0.0215 <sup>a</sup>	0.9212~0.9571
24 h control group	0.5960±0.0128	0.5854~0.6067
24 h liver failure group	1.1501±0.0278 <sup>b</sup>	1.1268~1.1733
24 h intervention group	1.1288±0.0317 <sup>b</sup>	1.1023~1.1552

Except for the control group SPP1 relative expression levels were higher in 24 h group than 12 h group. Liver failure and intervention group were increased compared with the control group (a: 12 h Intervention group or 12 h liver failure group vs 12 h control group,  $P<0.05$ ; b: 24 h Intervention group or 24 h liver failure group vs 24 h control group,  $P<0.05$ ). The intervention group compared with the model group showed no significant difference.

sis ( $R_{IL-6}=0.863$ ,  $R_{TNF-\alpha}=0.781$ ,  $P<0.01$ , two-tailed test) showed positive correlation.

### Liver gross morphology and pathology

After treatment, liver in liver failure group and intervention group lost luster surface (the surface was scattered and small punctuate hemorrhages) at 12 h.

Liver failure group and intervention group were diffuse bruising and bleeding at 24 h. In control group there are complete organizational liver lobule, clear hepatic cords arranged radially, no liver cell degeneration and necrosis (Figure 1 12 h-A, 24 h-A); He staining showed spotty necrosis, lymphocyte infiltration, sinusoidal and clear hepatic cords in liver failure group at 12 h (Figure 1 12 h-B). Liver necrosis, sinusoidal bleeding, disorders cord morphology were visible at 24 h in liver failure group (Figure 1 24-B), relatively mild liver disease, relatively

complete hepatic cords (Figure 1 24-C) were seen Intervention group.

### TUNEL test results

The dark brown apoptotic cells Stained by diaminobenzidine (DAB) and fluorescently labeled apoptosis increased in liver failure group and intervention group compared with the control group. Intervention group decreased (Figures 2 and 3) compared with liver failure group. Except for the control group the apoptosis in 24 h were higher than 12 h (Table 4) ( $P<0.05$ ). Apoptotic index and ammonia values were positively correlated by Pearson correlation coefficient 0.740 ( $P<0.01$ , two-tailed test).

### DNA Ladder electrophoresis

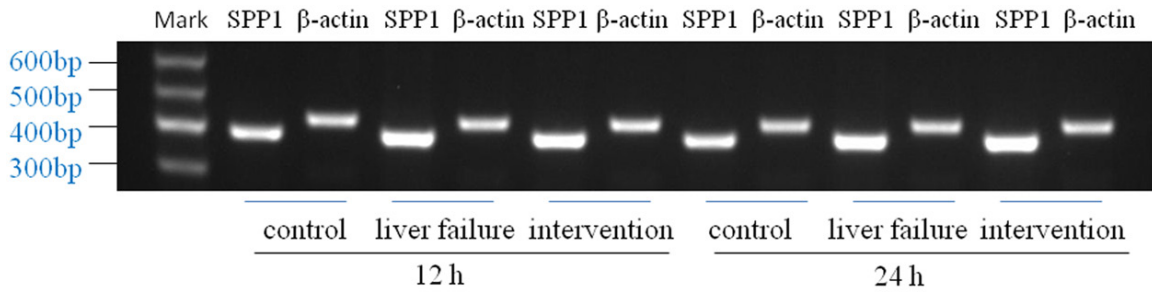
DNA Ladder is an important criterion for judging apoptosis, as seen in Figure 4 in addition to the control group, apoptotic cell-specific DNA Ladder can be seen in the other groups.

### RT-PCR results

P53 relative expression levels were increased in liver failure group and intervention group compared with the control group. Intervention group decreased (Table 5; Figure 5) compared with liver failure group. Except for the control group the apoptosis in 24 h were higher than 12 h ( $P<0.05$ ). Apoptotic index and ammonia values were positively correlated by Pearson correlation coefficient 0.740.

Except for the control group SPP1 relative expression levels were higher in 24 h group than 12 h group. Liver failure and intervention

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**Figure 6.** Electrophoresis of SPP1 gene amplification product. Liver failure and intervention group were increased compared with the control group.

group were increased compared with the control group ( $P<0.05$ ). The intervention group compared with the model group showed no significant difference (**Table 6; Figure 6**). SPP1 expression and ammonia value were positively correlated 0.703 ( $P<0.01$ , two-tailed test).

### Discussion

In this study, we established ALF rat model by treatment with D-galactosamine and endotoxin and applied ornithine aspartate (Yobo Division) to reduce blood ammonia. Serum samples and liver histology were analysed to study the impact of liver injury and apoptosis after lowering ammonia. Experimental results showed that blood ammonia had elevated in acute liver failure. As the ammonia values decreased after intraperitoneal injection of ornithine aspartate ammonia, the liver enzymes indicators (ALT, AST) decreased significantly and liver injury alleviated by pathology. Liver damage is aggravated with time but the value of serum ammonia decreased in 24 h compared with 12 h. The liver couldn't metabolize the excess ammonia in a timely manner which cause a sudden rise in blood ammonia value in acute liver cell injury. High concentrations of blood ammonia increased the compensatory ability and blood ammonia concentration decreased. When the body is in state of high blood ammonia, kidney will correspondingly increase glutamine production into the blood circulation to lower blood ammonia [17, 18]. Liver damage aggravate with elevated blood ammonia although the value of ammonia declined in 24 h, but still significantly higher than normal blood ammonia. Liver failure group showed no significant differences in serum markers (ALT, AST) and HE staining with intervention group at 12 h, probably because of the shorter administration time and the relatively low sensitivity of the indicators [19].

These results indicate that acute liver failure in rats given early lower ammonia treatment can reduce serum ammonia and delay the liver damage progress.

With prolonged hepatic failure and excessive accumulation of ammonia, IL-6 and TNF- $\alpha$  expression levels increased. IL-6 and TNF- $\alpha$  decreased after lowering blood ammonia at 12 h, which suggest that IL-6 and TNF- $\alpha$  were higher sensitivity than liver enzyme related indicators. When excessive endotoxin cause liver injury, hepatic stellate cells (HSC) secrete a variety of cytokines such as TNF- $\alpha$ , IL-6, etc. which can cause liver cell apoptosis and inflammatory cell infiltration and further damage to liver cells [20-22], these form a vicious spiral. As the ammonia decreased the TNF- $\alpha$ , IL-6 and other inflammatory cytokines also reduced and postponed disease progression.

DNA Ladder resulting from DNA fragment produced by apoptosis in gel electrophoresis is one of the characteristic indicators of apoptosis [23]. DNA Ladder appear in the rat except the control group. Meanwhile end labeling (TUNEL) showed hepatocyte apoptosis increased with D-galactosamine endotoxin stimulation. At the same time giving blood ammonia lowering drugs ammonia and hepatocyte apoptosis index were reduced. These results suggest that hepatocyte apoptosis is an important pathological changes in acute liver failure. Reducing the ammonia can reduce apoptosis in liver cells and liver injury. So ammonia may be an important contributing factor to aggravate liver cell damage by promoting apoptosis.

P53 gene is low expression in healthy cell [24]. P53 gene expression levels increased in each experimental group except the control group,

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lowering ammonia intervention can cause the expression of P53 decline and associated with blood ammonia levels. Tip hyperammonemia may induce high expression of P53 gene and induce apoptosis, while lowering blood ammonia can reduce liver cell DNA damage and inhibit the expression of the P53 gene and induce apoptosis.

Through our previous study, SPP1 (Osteopontin, OPN) maybe the most important gene expressed as hyperammonemia attacks. These suggest that it may be a key gene involved in liver damage [12]. SPP1 is an arginine-glycine-aspartic acid classified as extracellular matrix proteins, It can promote cell adhesion and migration [25, 26]. These results further validate the expression of SPP1 increased with the severity of liver failure. SPP1 expression decreased by lowering blood ammonia but no statistically significant difference, possibly because of shorter intervention time. These results further confirmed the regulation effect of gene SPP1 on the liver cell in acute liver failure, and may.

In short, lowering blood ammonia may reduce liver injury and hepatocyte apoptosis in the ALF rat model. Serum IL-6 and TNF- $\alpha$  maybe more sensitive indicators to assess liver injure than liver serum ALT, AST. SPP1 maybe another sensitive indicators for liver injure and more associated with ammonia.

### Disclosure of conflict of interest

None.

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