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Protection of plasma transfusion against lipopolysaccharide/D-galactosamine-induced fulminant hepatic failure through inhibiting apoptosis of hepatic cells in mice*

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Abstract: Fulminant hepatic failure is a severe clinical condition associated with extremely poor outcomes and high mortality. A number of studies have demonstrated the ability of plasma transfusion to successfully treat fulminant hepatic failure, but the underlying mechanisms are not well understood. The aim of the present study is to define the mechanisms of plasma transfusion treatment in lipopolysaccharide/D-galactosamine (LPS/D-GalN)-induced mice. LPS/D-GalN treatment in mice causes significant hepatic failure, including increasing serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels, histopathological changes in centrilobular necrosis and inflammatory cells, and the up-regulation of inflammation (tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6)). When LPS/D-GalN-induced mice were treated with plasma, these changes were halted. Results showed that plasma transfusion significantly reduced mortality, and decreased the levels of AST, ALT, and inflammation factors such as TNF-α and IL-6. The expression levels of cleaved Caspase-3, BAX, and p53 were down-regulated and Bcl-2 was up-regulated, suggesting that plasma can reduce LPS/D-GalN-induced apoptosis. The protective mechanism of plasma against LPS/D-GalN-induced fulminant hepatic failure is related to the inhibition of the inflammatory response and the reduction in apoptosis through the down-regulation of the p53-induced apoptotic pathway.

Key words: Fulminant hepatic failure; Plasma; Inflammation; Apoptosis
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1 Introduction

Fulminant hepatic failure develops rapidly and can result in an overproduction of various toxins, which can ultimately lead to multi-organ failure. Without appropriate treatment, severe, irreversible hepatitis can be developed (Kaplowitz, 2000; Kamimura et al., 2014). Many strategies have been used to treat severe hepatitis, including medication, such as antivirals and growth factor drugs and drugs aimed at protecting the liver, decreasing enzymes, and eliminating jaundice, liver transplant, artificial liver, and

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plasma exchange. Plasma exchange involves leading the patient's blood outside the body, separating the plasma, and replacing it with the same amount of fresh plasma or human serum albumin, which removes metabolic toxins and pathogenic factors and adds beneficial inflammatory factors (Nakamura et al., 2000; Jayne et al., 2007). Plasma exchange has been reported as a useful extracorporeal treatment in fulminant hepatic failure (Iwai et al., 1998). However, there has been little research on the efficacy of plasma transfusion, which is quite different from plasma exchange.

Plasma, the liquid isolated from the whole blood, contains a variety of plasma proteins, low-molecular-weight compounds, and coagulation factors. Traditionally, plasma transfusion has been used in clinical therapy to correct a deficit in coagulation factors accompanied by bleeding in, typically, adjunctive therapy empyrosis, hemophilia, and other diseases (Havens et al., 2016; MacLennan, 2016). Recent research has suggested that plasma transfusion has been used as the main treatment for severe alcoholic hepatitis (Horie, 2012). Mintz et al. (2006) reported that plasma is important for patients with acquired coagulopathy through down-regulation of prothrombin time (PT) and partial thromboplastin time (PTT). These patients frequently have end-stage liver disease and require large volumes of plasma during a single treatment. Youssef et al. (2003) found that suitable plasma transfusion and higher volumes of plasma (6 or more units) may be more effective at correcting the prolonged PT of chronic liver disease. However, the underlying mechanisms of plasma transfusion on hepatic injury remain unknown, particularly on inflammation and apoptosis.

Lipopolysaccharide (LPS) can cause massive release of inflammatory mediators. D-Galactosamine (D-GalN), a specific hepatotoxic drug, can obstruct the synthesis of cellular RNA and protein. Both would rapidly cause apoptosis, necrosis, and diffuse inflammation of liver cells. LPS/D-GalN-induced acute hepatic injury in mice is a typical fulminant hepatic failure model, which can mimic the activation of monocyte-macrophage cells, releasing various inflammatory factors and resulting in the apoptosis and necrosis of liver cells (Gu et al., 2014; Xia et al., 2014).

This study investigated the effect of plasma transfusion on fulminant hepatic failure and the mechanisms involved using an LPS/D-GalN-induced mice model.

2 Materials and methods

2.1 Reagents

LPS and D-GalN were purchased from Sigma (St. Louis, MO, USA). Compound glycyrrhizin was purchased from Beijing Kain Science and Technology Co., Ltd. (Beijing, China). Hematoxylin-eosin (HE) staining techniques such as hematoxylin, eosin, neutral gum, and other related supplies were from Beijing Zhongshan Golden Bridge Biotechnology (Beijing, China). Mouse enzyme-linked immunosorbent assay (ELISA) kits (tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6)) and alanine aminotransferase (ALT), aspartate aminotransferase (AST) kits were from Nanjing Jiancheng (Nanjing, China). Phenylmethanesulfonyl fluoride (PMSF), the nuclear and cytoplasmic protein extraction kit, and bicinchoninic acid were from Beyotime (Shanghai, China). The in situ apoptosis detection kit was from Roche Diagnostics (Shanghai, China). P53, cleaved Caspase-3, Bcl2-associated X protein (BAX), and Bcl-2 were from Cell Signaling Technology (Danvers, MA, USA); and β-actin was from Abcam (Cambridge, UK).

2.2 Animals

Pathogen free ICR mice (6–8 weeks old) were obtained from the Laboratory Animal Center of Zhejiang Province, China (Certification No. SCXK 2008-0033). The mice were housed in standard conditions including light (12-h light/dark cycle), temperature ((22±2) °C), and humidity ((50±5)%). All animals had free access to standard laboratory food and water. The Animal Care and Use Committee of the School of Medicine, Zhejiang University approved all animal experiments.

2.3 Experimental groups

Mice were simultaneously injected intraperitoneally with LPS (50 μg/kg) and D-GalN (500 mg/kg) dissolved in phosphate-buffered saline (PBS). Animals were randomly divided into four groups with ten mice in each group: (1) control group: mice were injected with the same volume of sterile saline solution; (2) plasma group: mice were injected with

plasma into a tail vein three times (150 μl each), 2, 4, and 8 h after the sterile saline injection; (3) LPS/D-GalN group: mice were given only LPS/D-GalN by intraperitoneal injection; (4) LPS/D-GalN+plasma group: mice were injected with plasma into a tail vein three times (150 μl each) 2, 4, and 8 h after the LPS/D-GalN injection. The mice were sacrificed by decapitation 12 h after LPS/D-GalN administration. Blood and liver samples were then quickly collected and frozen at −80 °C for biochemical and histological analyses. The survival analysis of mice was monitored for 32 h after LPS/D-GalN injection.

2.4 Measurement of serum aminotransferase activitiy

Collected blood samples were centrifuged at 3000 r/min for 10 min to obtain serum samples, which were hemolysis-free and were stored at 4 °C before use. The serum levels of ALT and AST were measured using ALT and AST kits.

2.5 Histology

The collected liver tissues were rinsed gently with PBS and preserved in 10% paraformaldehyde. The samples were then dehydrated and embedded in paraffin. The samples were sectioned (5 μm thick) and stained with HE and reticular fiber. Histological changes including necrosis, hemorrhage, and inflammation were graded with a five-point severity scale of "−" to "+++" (−: no change; ±: slight change; +: mild change; ++: moderate change; +++: strong change) in accordance with the literature (Fukuda et al., 2006).

2.6 Cytokine assays

Two hours after the animal was sacrificed, a 0.1-g liver tissue sample was extracted and washed by 1× PBS three times. The supernatant was collected by centrifuging at approximately 3000 r/min for 25 min. The concentrations of cytokines (TNF-α and IL-6) in liver tissue were assayed using a mouse ELISA kit according to the manufacturer's instructions. The sensitivity of the kit was 1.0 ng/ml.

2.7 TUNEL assay

Apoptosis in cells in the liver tissue sections was detected using an in situ apoptosis detection kit (Roche Diagnostics). Liver tissue sections were rinsed with double-distilled water (ddH_2O), xylene-dewaxed, gradient ethanol-hydrated, and treated with proteinase K. After using the deoxyribonucleotidyl transferase (TDT)-mediated dUTP nick end labeling (TUNEL) reaction mixture and the converter-peroxidase (POD), the sections were induced with the horseradish peroxidase (HRP) substrate 3,3'-diaminobenzidine (DAB). Finally, sections were counterstained with hematoxylin. Positive cells were identified and counted (three random fields per slide) under light microscope (Carl Zeiss, Thornwood, NY, USA).

2.8 Western blot

Proteins were extracted by lysing liver tissue with radio immunoprecipitation assay (RIPA) lysate containing 1% PMSF following the manufacturer's directions. The concentration of proteins was quantified using a bicinchoninic acid (BCA) protein assay kit. Equal amounts of proteins were loaded and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and were electrotransferred onto polyvinylidene difluoride (PVDF) membranes. The blots were blocked with 5% non-fat milk for 1 h and probed with specific primary antibodies against IL-6 (1:1000 diluted), BAX (1:1000 diluted), cleaved Caspase-3 (1:1000 diluted), p53 (1:1000 diluted), and Bcl-2 (1:1000 diluted) at 4 °C overnight and subsequently incubated at 37 °C with their corresponding secondary antibodies (1:5000 dilution) for 45 min. Unbound antibodies in each step were washed three times by Tris-buffered saline and Tween 20 (TBST). Target bands were visualized by enhanced chemilumescent (ECL) solution and measured by Gel-Pro-Analyzer software. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was employed as an internal control.

2.9 Data analysis

The statistical analysis was performed using Prism 6.0, and measurement data were expressed by mean±standard error of the mean (SEM). The differences between groups were assessed using one-way analysis of variance (ANOVA) followed by Tukey's test, in which $P<0.05$ was considered statistically significant. Survival rates were analyzed using the Kaplan-Meier method and log-rank test.

3 Results

3.1 Effects of plasma transfusion on survival and serum levels of aminotransferase

Mice began to die 12 h after LPS/D-GalN injection, with mortality reaching 90% at 32 h (Fig. 1). There was no significant difference in the mortality between the control and plasma groups. The mortality rate was decreased by LPS/D-GalN+plasma to 40% (4/10). The activity of ALT and AST in serum was assessed to evaluate the severity of LPS/D-GalN-induced hepatic failure (Fig. 2). ALT and AST in serum are important biochemical indicators of stem cell damage. There were no significant differences in the levels of the ALT and AST between the plasma group and the control group, but there was a significant increase (P<0.05) in ALT and AST serum levels in the LPS/D-GalN group, with levels reaching (125.01±12.07) U/L and (288.10±32.14) U/L, respectively. This indicated that the model of fulminant hepatic failure was successful. After plasma treatment, the levels of ALT and AST were significantly inhibited (P<0.05) compared with the LPS/D-GalN group to (86.31±8.98) U/L and (181.48±35.02) U/L, respectively.

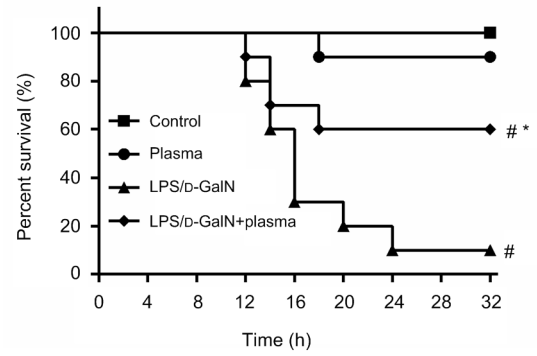

Fig. 1 Effect of plasma on the survival rate of LPS/D-GalN-induced mice

The survival rate was monitored for 32 h after LPS/D-GalN injection. Survival curves were obtained using the Kaplan-Meier method and were compared using the log-rank test. # P<0.05, vs. the control group; * P<0.05, vs. the LPS/D-GalN group

3.2 Histopathological analysis

In the control and plasma groups, histological observation showed normal cell structure and liver lobular architecture. The liver was red in color, had a smooth surface and delicate texture, with no sign of infiltration by inflammatory cells. The histological appearance of liver tissue in the LPS/D-GalN group revealed marked morphological changes, including disordered liver cell cord, a widened sinusoidal gap with marked necrosis and inflammatory cell infiltration. Liver damage in the LPS/D-GalN+plasma group was significantly less severe (Fig. 3). Plasma significantly reduced the histological grading of hepatic failure induced by LPS/D-GalN in mice (Table 1).

3.3 Effect of plasma transfusion on cytokine production induced by LPS/D-GalN

The underlying mechanisms influencing the regulatory effects of plasma on the levels of multiple

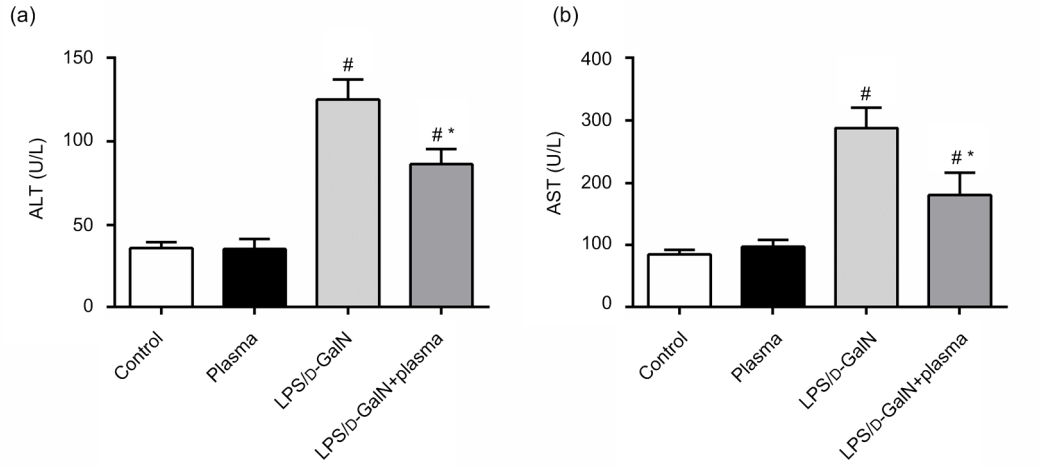

Fig. 2 Effect of plasma on serum AST and ALT activity in LPS/D-GalN-induced mice

(a) Serum ALT levels; (b) Serum AST levels. The values are presented as mean±SEM (n=10). # P<0.05, vs. the control group; * P<0.05, vs. the LPS/D-GalN group

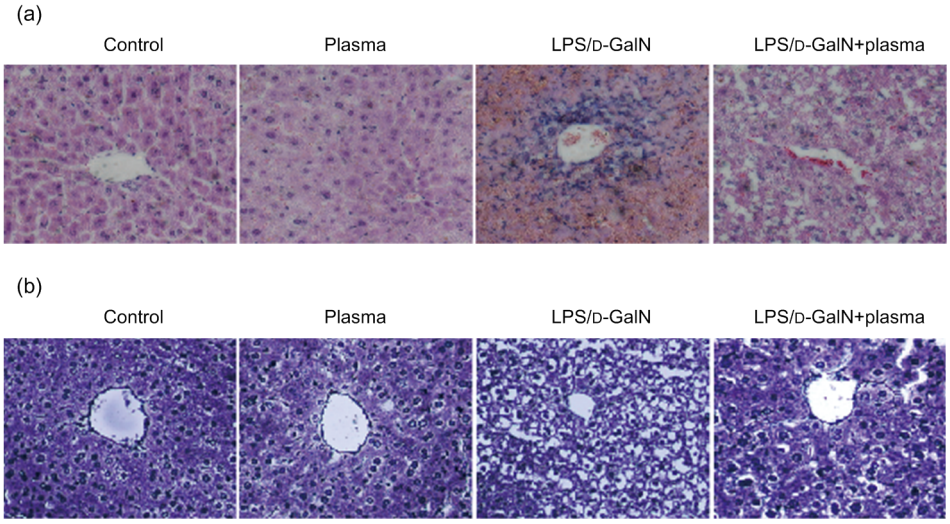

Fig. 3 Effect of plasma on the histopathological change in LPS/D-GalN-induced mice

(a) Hematoxylin-eosin staining (original magnification ×200); (b) Reticular fiber staining (original magnification ×200). The control group shows normal histopathological structure of liver. The plasma group shows the same as the control group. The LPS/D-GalN group shows markedly hepatic failure, especially multiple and extensive areas of hepatocellular necrosis and inflammatory cell infiltration. The LPS/D-GalN+plasma group shows minimal hepatocellular necrosis and inflammatory cell infiltration

Table 1 Histological grading in hepatic failure by LPS/D-GalN exposure in mice

Group (n=10 each group)	Histological grading				
	–	±	+	++	+++
Control	10	0	0	0	0
Plasma	9	0	1	0	0
LPS/D-GalN	0	0	2	2	6
LPS/D-GalN+plasma	0	0	3	5	2

inflammatory cytokines and mediators were investigated using ELISA. Tissue levels of the pro-inflammatory cytokines, TNF-α and IL-6, were significantly increased (P<0.05) by LPS/D-GalN injection compared with the controls. The injection of the LPS/D-GalN+plasma significantly decreased (P<0.05) the elevations of TNF-α and IL-6 compared with the LPS/D-GalN group (Fig. 4).

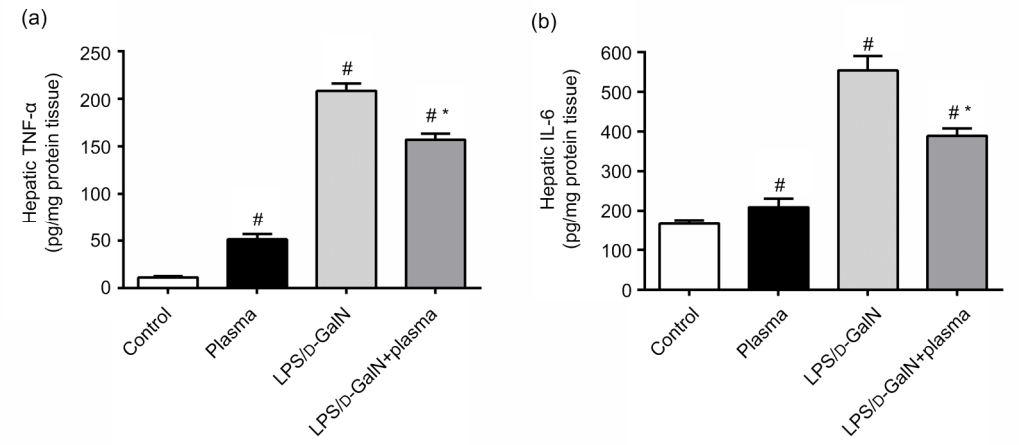

Fig. 4 Effects of plasma on the tissue levels of cytokines in LPS/D-GalN-induced mice

(a) Hepatic TNF-α expression levels; (b) Hepatic IL-6 expression levels. The values are presented as mean±SEM (n=5). $^{\#}$ P<0.05, vs. the control group; * P<0.05, vs. the LPS/D-GalN group

3.4 Effect of plasma transfusion on apoptosis induced by LPS/D-GalN

To further examine whether plasma transfusion affects the survival of hepatocytes, we performed a TUNEL staining assay. As shown in Fig. 5a, TUNEL positive cells were significantly decreased after treatment by plasma transfusion, compared with the LPS/D-GalN group. Western blot results showed that the level of cleaved Caspase-3 was significantly decreased (Fig. 5c), Bcl-2 (anti-apoptosis protein) increased (Fig. 5d), and pro-apoptosis protein, BAX, and p53 decreased after plasma treatment (Figs. 5e and 5f) compared with the LPS/D-GalN group. These results strongly suggested that plasma transfusion had a protective effect on the apoptosis of hepatic cells through the p53-induced apoptotic pathway in LPS/D-GalN-induced mice.

4 Discussion

Fulminant hepatic failure is associated with high mortality and characterized by significant necrosis of

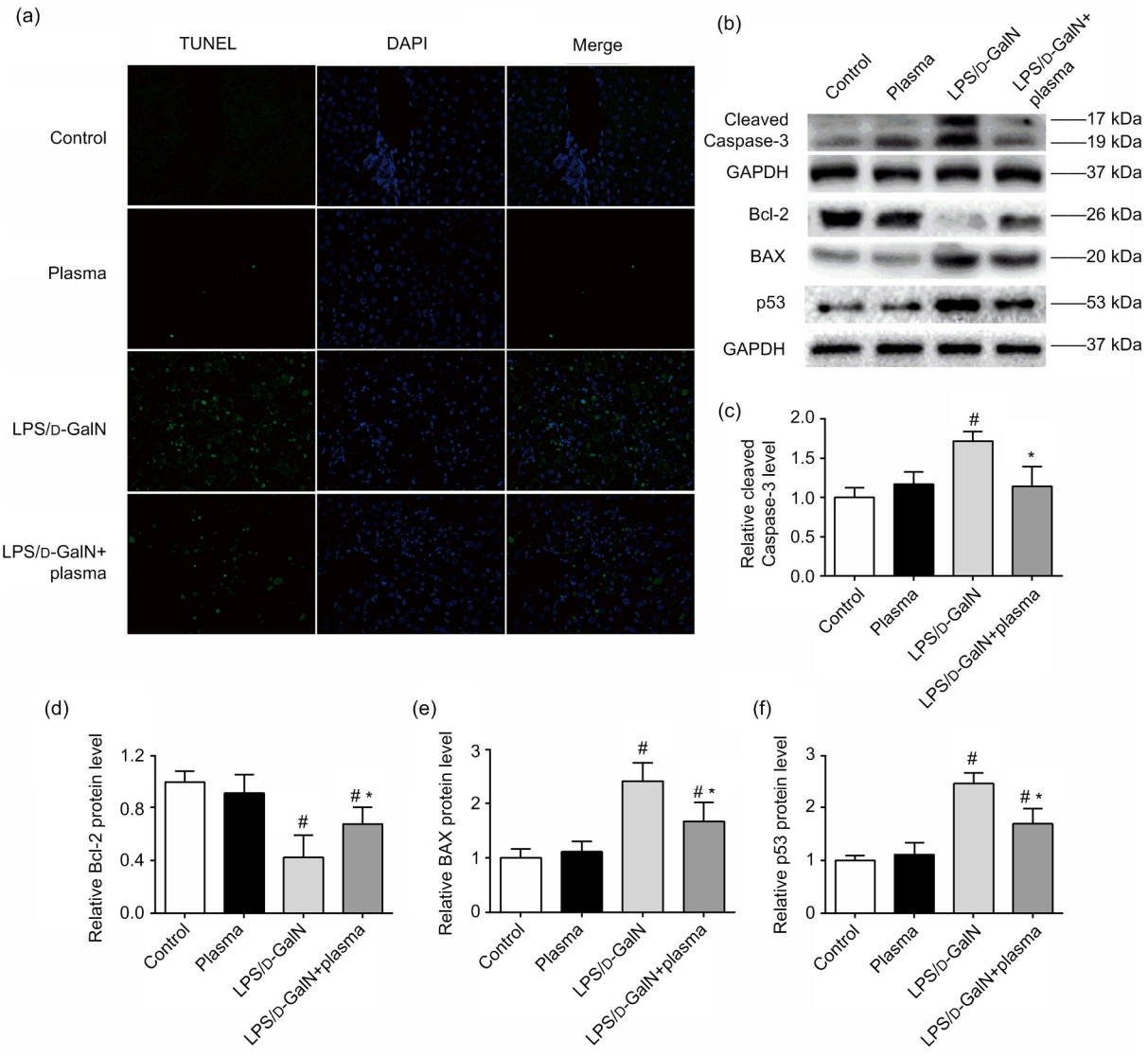

Fig. 5 Effect of plasma transfusion on apoptosis induced by LPS/D-GalN

(a) TUNEL assay detected the apoptosis of liver tissue (original magnification ×400); (b) Western blot detected the downstream signaling, cleaved Caspase-3, Bcl-2, BAX and p53; (c–f) Quantitative analyses of the relative cleaved Caspase-3 (c), Bcl-2 (d), BAX (e), and p53 (f). The values normalized to control are presented as mean±SEM (n=3). $^{\#}$ P<0.05, vs. the control group; * P<0.05, vs. the LPS/D-GalN group

liver cells, high levels of transaminase, and the activation of inflammation mediators including IL-10, IL-6, and TNF-α (Fukuda et al., 2006; Kim et al., 2014). Plasma transfusion has recently been recognized as a powerful clinical treatment for fulminant hepatic failure by correcting inflammatory mediators and blood coagulation function, although the mechanism of action has not been clear (Mintz et al., 2006). LPS and D-GalN-induced hepatic failure in mice is widely used as an experimental model of fulminant liver failure, producing a similar histopathological phenotype and immune alteration as in clinical symptoms (Chen et al., 2016). This study demonstrated the protective effect of plasma transfusion against LPS/D-GalN-induced fulminant hepatic failure and the underlying mechanism by which plasma transfusion inhibits apoptosis of hepatic cells in mice.

The dosage of LPS/D-GalN was 50 μg LPS and 500 mg D-GalN per kg body weight. With this treatment, mice began to die after 12 h, the mortality rate reaching 90% after 32 h (Fig. 1). Increased levels of ALT and AST, which are involved in the metabolism of human proteins, indicate hepatic structural damage and are the typical symptoms of fulminant hepatic failure (Nakasone et al., 2001; Kamimura et al., 2014). The increases of ALT and AST in the LPS/D-GalN group are the same as in previous observations (Fig. 2) (Chen et al., 2016). From histopathology, we observed extensive necrosis and inflammatory cell infiltration in the LPS/D-GalN group. Plasma transfusion reduced ALT and AST enzyme activity (Fig. 2) and changed the histopathology (Fig. 3).

Another pathological effect of plasma transfusion involves alteration of pro-inflammatory cytokines (Nakasone et al., 2001). The development of fulminant hepatic failure activates inflammatory cells and then stimulates an excess release of pro-inflammatory cytokines including IL-6, TNF-α, IL-10, and IL-1β (Ma et al., 2015). In contrast to other pro-inflammatory cytokines, TNF-α is considered a major inflammatory cytokine, involved in the inflammatory cascade by the inhibition of IL-10 and IL-6 (Ramesh and Reeves, 2002; Jiang et al., 2012; Yan et al., 2016). Anti-inflammatory factors may therefore be a target for the treatment of hepatic failure (Wang et al., 2016). In the present study, we found that plasma transfusion markedly inhibited TNF-α and IL-6 levels (Fig. 4). These results suggested that plasma transfusion has the effect of inhibiting the inflammatory response in LPS/D-GalN-induced mice.

Apoptosis is a complicated biological process that regulates proliferation of fulminant hepatic cell division and death (Zhang L et al., 2016). Necrotic hepatocytes promote hepatocellular apoptosis by activating apoptotic signaling pathways, such as Caspase and the Bcl-2 families (Xia et al., 2014). Our present study pointed out that LPS/D-GalN could induce cell apoptosis and revealed that hepatic cell apoptosis could be reduced by plasma transfusion (Fig. 5). Thus, down-regulating hepatic cell apoptosis may block the detrimental implications of fulminant hepatic failure. The primary intracellular regulation of cell apoptosis is through the activation of cysteine proteases, particularly Caspase (Mu et al., 2010). Detection of cleaved Caspase-3, which causes cell apoptosis, confirms cell apoptosis (Zhang Q et al., 2016). The results demonstrated that cleaved Caspase-3 was increased significantly in LPS/D-GalN group, indicating that hepatic cells were undergoing apoptosis. Further, a down-regulated cleaved Caspase-3 was demonstrated in the LPS/D-GalN and plasma transfusion groups, but there was no alteration in apoptosis in plasma transfusion alone. These results indicated that plasma transfusion may protect hepatic cells from apoptosis.

In order to further clarify the mechanism, we checked the p53-dependent apoptotic signaling pathway. Several studies have reported that p53 directly binds to the Bcl-2 protein family involved in the cytotoxic effect of cell apoptosis through the intrinsic pathway (Galehdar et al., 2010; Liu et al., 2016). According to previous reports, p53 promotes the transcription of BAX and BAK which regulates mitochondrial functions and results in cell apoptosis (Ikeda et al., 2000; Zhang L et al., 2016). In our study, we observed a significant difference in the inhibition of p53. Plasma transfusion induced up-regulation of Bcl-2 and down-regulation of BAX (Fig. 5). These results implied that plasma transfusion has a beneficial clinical application in the treatment of fulminant hepatic failure patients. Meanwhile, plasma is a complex mixture, which has many components, so, identification of which component has the strongest effects on hepatic cells needs further investigation.

In summary, the protective effect of plasma transfusion in a mouse model of LPS/D-GalN-induced

hepatic failure was established. We have demonstrated that plasma transfusion inhibits excessive inflammation and apoptosis in LPS/D-GalN-induced mice mediated by the p53-dependent apoptotic signaling pathway.

Contributors

Bing-yu CHEN and Lu-xi JIANG performed the experimental research and data analysis, wrote and edited the manuscript. Ke HAO, Lu WANG, and Ying WANG performed the data analysis and wrote the paper. Yi-wei XIE and Jian SHEN performed the establishment of animal models. Meng-hua ZHU and Xiang-ming TONG collected and analyzed the data. Kai-qiang LI and Zhen WANG contributed to the study design, data analysis, writing and editing of the manuscript. All authors read and approved the final manuscript and, therefore, had full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Bing-yu CHEN, Lu-xi JIANG, Ke HAO, Lu WANG, Ying WANG, Yi-wei XIE, Jian SHEN, Meng-hua ZHU, Xiang-ming TONG, Kai-qiang LI, and Zhen WANG declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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中文概要

题　目：血浆输注通过抑制肝细胞凋亡对脂多糖/D-半乳糖诱导的小鼠急性肝损伤的保护作用

目　的：评估血浆输注对脂多糖/D-半乳糖（LPS/D-GalN）诱导的小鼠急性肝损伤的保护作用，并探讨其作用机制。

创新点：在小鼠急性肝损伤模型中证明血浆输注对肝损伤的保护作用，且此作用与 p53 介导的肝细胞凋亡相关。

方　法：将 40 只清洁型 ICR 雄性小鼠随机分为 4 组（n=10 每组）：（1）对照组；（2）血浆（plamsa）组；（3）LPS/D-GalN 组；（4）LPS/D-GalN+plamsa 组。收集血清和肝组织样本，用天门冬氨酸转氨酶（AST）和丙氨酸氨基转移酶（ALT）试剂盒检测血清中 AST 和 ALT 水平；用酶联免疫吸附法（ELISA）检测肝组织中白细胞介素-6（IL-6）和肿瘤坏死因子-α（TNF-α）的表达变化；肝组织进行苏木精-伊红染色法（HE）和网状纤维染色，显微镜下观察肝组织病理学变化；用免疫印迹法（WB）检测凋亡相关蛋白的变化。在腹腔注射 LPS/D-GalN 后 32 小时内对小鼠进行存活分析。

结　论：LPS/D-GalN 能够显著诱导小鼠的急性肝损伤，包括增加血清中 AST 和 ALT 水平；中心小叶坏死和炎性细胞的组织病理学变化和炎症上调（TNF-α 和 IL-6）。当血浆输注后，这些变化被缓解。结果显示，血浆输注显著降低小鼠死亡率，降低 AST、ALT 和炎症因子如 TNF-α 和 IL-6 的水平。Cleaved Caspase-3、BAX 和 p53 的表达下调，Bcl-2 上调，表明血浆可以减少 LPS/D-GalN 诱导的细胞凋亡。血浆输注对 LPS/D-GalN 诱导的急性肝损伤的保护机制与通过 p53 诱导的凋亡途径和炎症因子减少相关。

关键词：肝损伤；血浆；炎症；凋亡