Original Article
A novel acute lethal liver injury mouse model with visualization of NF-κB activity for treatment of severe acute liver injury

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Abstract: Acute lethal inflammation, especially that related to liver injury, is an important clinical issue. To date, however, there is no model that can be used to assess this serious condition. This study was designed to establish a novel lipopolysaccharide (LPS)/D-galactosamine (D-GalN)-induced acute lethal liver injury model in nuclear factor-κB (NF-κB) transgenic mice. The results show that a high dose of LPS (500 µg/kg) plus D-GalN (800 mg/kg) successfully established a novel mouse model of acute lethal liver injury with a lifespan of 8-10 h. Significantly increased NF-κB activity, detected with an *in vivo* imaging system (IVIS), peaked at approximately 4 h post-LPS/D-GalN challenge in NF-κB transgenic mice. Moreover, the serum levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6, and monocyte chemoattractant protein (MCP)-1 were significantly increased and peaked at approximately 4 h post-*i.p.* injection of LPS/D-GalN. The serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) also sharply increased. Correlation analyses showed that NF-κB activity was significantly correlated with serum levels of ALT and AST. The mouse model livers showed marked congestion and hemorrhage, and hematoxylin and eosin (H&E) staining confirmed the destruction of the lobular structure and severe hepatocyte necrosis and hemorrhage. None of these changes were observed in the control mice. In summary, a novel LPS/D-GalN-induced acute lethal liver injury model with visualization of NF-κB activity was established in NF-κB transgenic mice. This model will provide the technology for developing new therapeutic strategies for treatment of severe acute liver injury complicated by endotoxemia or septicemia.

Keywords: Liver injury, lipopolysaccharide/galactosamine (LPS/D-GalN), nuclear factor-κB (NF-κB), alanine aminotransferase (ALT)

Introduction

Acute lethal inflammation, especially in liver injury, is an important clinical issue. The lipopolysaccharide (LPS)/galactosamine (D-GalN)-induced acute liver injury mouse model has been extensively utilized for more than 20 years [1-6] and correlates with human acute liver injury complicated by endotoxemia or septicemia [4, 7, 8]. However, there is still no such a model that can be used for studies on the serious condition of acute lethal inflammation.

The serum levels of alanine transaminase (ALT) and aspartate transaminase (AST) increase significantly in the acute liver injury mouse model. Furthermore, the pathology is characterized by a disordered hepatic leaflet structure, dilated

central vein, focal necrosis, and large spots of hemorrhage and inflammatory cell infiltration [6, 9, 10]. These data are in accordance with the characteristics of acute liver failure in human clinical practice. In addition, LPS induces the release of a large number of inflammatory mediators, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-2 [4, 11]. These inflammatory mediators can trigger a series of pathological and biological processes, resulting in pathological changes such as pyrexia, shock, and disseminated intravascular coagulation [12]. D-GalN also triggers adverse effects by directly exhausting uridylic acid in liver cells, which subsequently induces the reduction of nucleic acids, glycoproteins, and glycolipidsfor uridylic acid-dependent biosynthesis, cellular regeneration, and production and supplemen-
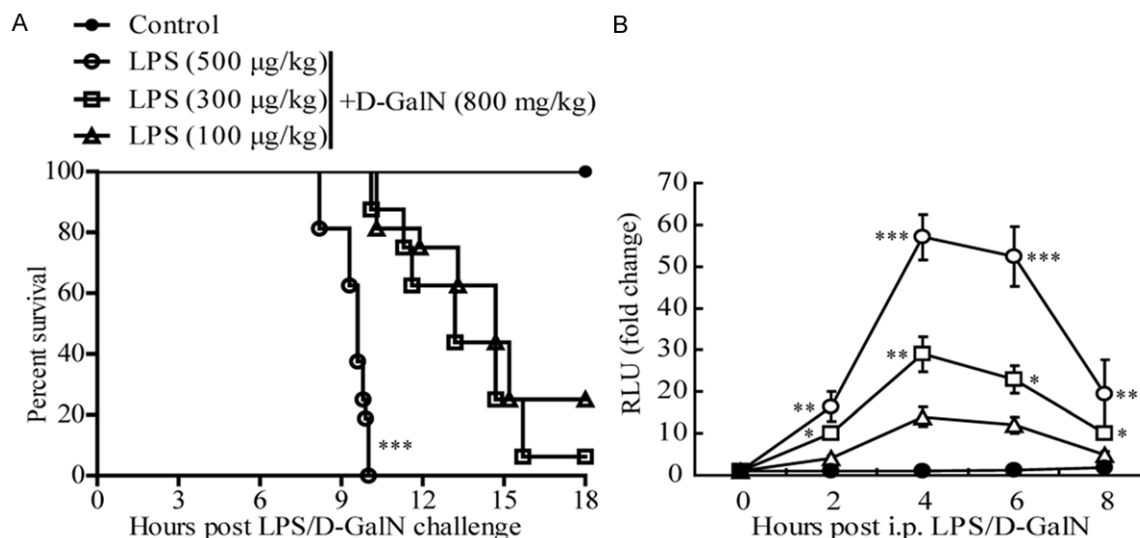
Figure 1. Survival and fluorescence intensity of NF-κB activity of acute lethal liver injury mice. NF-κB transgenic mice were given *i.p.* injections of LPS (100 µg/kg)+D-GalN (800 mg/kg), or LPS (300 µg/kg)+D-GalN (800 mg/kg), or LPS (500 µg/kg)+D-GalN (800 mg/kg) to establish an acute lethal liver injury model. Control group mice were given *i.p.* injections of an equal volume of saline. A. Survival observations. B. Fluorescence intensity of NF-κB activity in different groups (n=16 mice per group). *$P<0.05$, **$P<0.001$, ***$P<0.001$.

tation of enzymes. These changes promote structural and functional abnormalities in liver cells. Moreover, D-GalN produces synergistic effects with LPS [6, 11]. Overall, these effects promote the outbreak and degranulation of neutrophils aggregating around injured liver cells [5], release oxygen free radicals and induce lipid peroxidation, act on liver parenchymal cells and vascular endotheliocytes, and result in severe cell injury or death [13].

Only one intraperitoneal (*i.p.*) injection of a certain dose of D-GalN is needed to induce an acute liver injury model. However, different doses of LPS may result in varied acute states and severities in models established using different animal strains. The varied acute states and severities manifest as different survival times, liver functions, and pathological changes [14-19].

In this study, an acute lethal liver injury model with visualization of nuclear factor-κB (NF-κB) activity induced by LPS/D-GalN in NF-κB transgenic mice was established. This model can be used for studies of acute liver injury complicated by endotoxemia or septicemia.

Materials and methods

Animals

NF-κB-RE-luc (Oslo) transgene mice (NF-κB transgenic mouse) were purchased from Xenogen

Corporation (Alameda, CA, USA). Animals were maintained in aspecific pathogen-free facility at the Experimental Animal Center, Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences. All experiments were approved by the Ethics Committee for Experimental Animals and performed according to the national guidelines for animal welfare.

Materials

LPS-2630 (*Escherichia coli* serotype 2630) and D-GalN were purchased from Sigma-Aldrich (St. Louis, MO, USA). The mouse cytokine cytometric bead array (CBA) kit was from Bender Medsystems (Burlingame, CA, USA). The ALT and AST assay kits were purchased from Nanjing Institute of Biological Engineering (Nanjing, Jiangsu, China). D-Luciferin was purchased from Promega Company (Madison, WI, USA).

Establishment of acute lethal liver injury mice model

NF-κB transgenic mice were divided into the following groups: acute lethal liver injury model group mice were *i.p.* injected with LPS (100 µg/kg)+D-GalN (800 mg/kg), LPS (300 µg/kg)+D-GalN (800 mg/kg), or LPS (500 µg/kg)+D-GalN (800 mg/kg). Control group mice were *i.p.* injected with equal volume of saline. Following
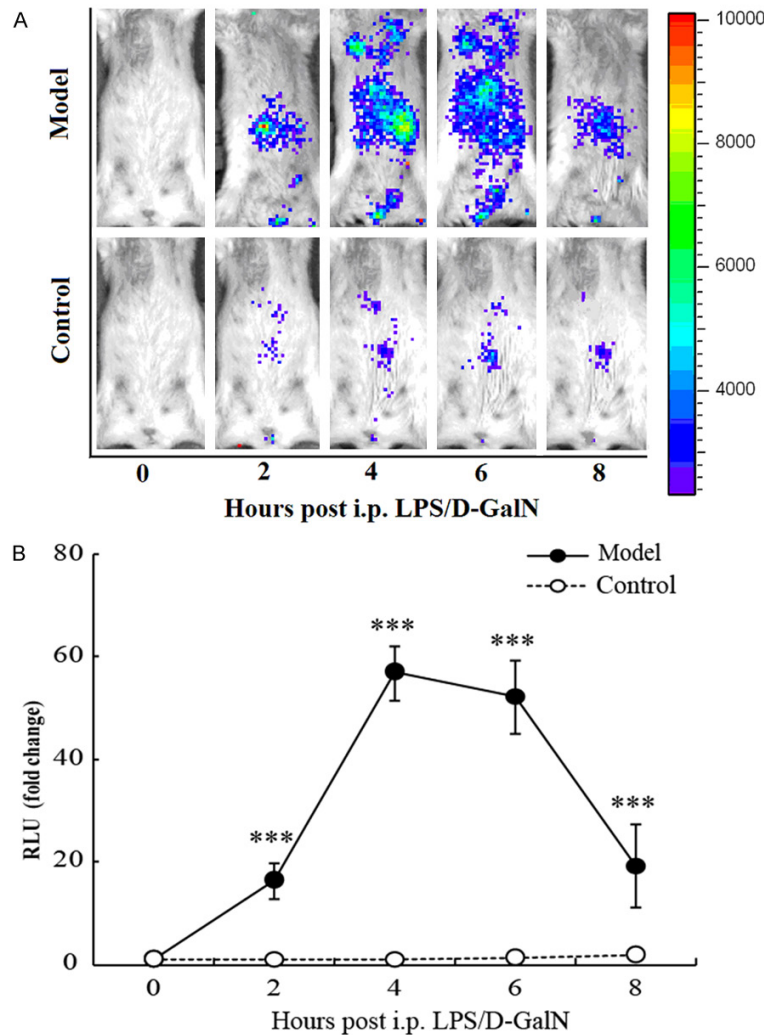
Figure 2. NF-κB activity in acute lethal liver injury mice. NF-κB-RE-luc (Oslo) luciferase reporter transgenic mice challenged with LPS (500 μg/kg)+D-GalN (800 mg/kg) or with saline as control. *In vivo* images were taken at 0, 2, 4, 6, and 8 h after the challenge. A. Representative pictures showing NF-κB activities measured as described in the methods. B. NF-κB activity over time. Data are expressed as means ± SD (*n*=6) of three independent experiments. ***$P<0.001$.

graphed, and analyzed using hematoxylin and eosin (H&E) staining.

Detection of NF-κB activity in acute lethal liver injury mice

After receiving *i.p.* injections of LPS+D-GalN at five different times (0, 2, 4, 6, and 8 h), NF-κB transgenic mice also received an *i.p.* injection of a luciferase substrate (D-amino phenol, 150 mg/kg in 250 μL saline). Control NF-κB transgenic mice also received an *i.p.* injection of a luciferase substrate. Animals were immediately transferred into the cage of the IVIS Imaging System 200 (Xenogen Corporation, Alameda, CA, USA) to be photographed. Three minutes later, luminous signals were counted as the number of collected released photons per 5 min. Control mice results were used as a baseline reference. The normalized luciferase activity is presented as fold change of relative light units (RLU).

Statistical treatment

Data are presented as means ± S.D. For comparisons between two groups, an unpaired two-tailed Student's *t*-test and Wilcoxon rank-sum test were used for parametric and nonparametric data, respectively. Multiple group comparison was performed using one-way ANOVA, followed by Bonferroni correction or Dunnett post-hoc tests. If significance was reached, an unpaired two-tailed Student's *t*-test was performed between each compared population, unless otherwise indicated. Survival curves were analyzed by the nonparametric Kaplan-Meier method. Correlation analyses of the relationships between NF-κB activity and serum levels of ALT and AST were made with scatter plots using SPSS 15.0 and Polynomial Curve Fitting (third order) from Microsoft Excel. Statistical analysis

treatment, mice were labeled and placed into cages for observation.

Samples collection and detection

Mice were sacrificed at 0, 2, 4, 6, and 8 h post *i.p.* injection of LPS+D-GalN, and then serum and liver specimens were collected. Flow cytometry was used to detect serum levels of TNF-α, IL-6, and MCP-1 using a CBA kit according to the supplier's instructions. Serum levels of ALT/AST were detected using an ALT/AST test kit. Isolated livers were then observed, photo-
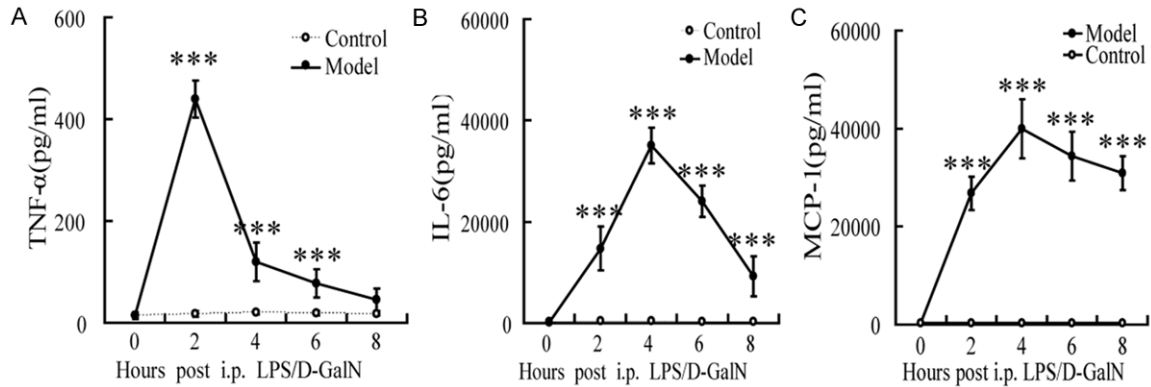
Figure 3. Inflammatory cytokines in acute lethal liver injury mice. NF-κB-RE-luc (Oslo) luciferase reporter transgenic mice challenged with LPS (500 μg/kg)+D-GalN (800 mg/kg) or with saline as control. Serum was collected at 0, 2, 4, 6, and 8 h after the challenge. Serum levels of (A) TNF-α, (B) IL-6. and (C) MCP-1 were detected with a commercial mouse cytokine CBA kit. Data are expressed as means ± SD (*n*=6) of three independent experiments. **P<0.01, ***P<0.001.
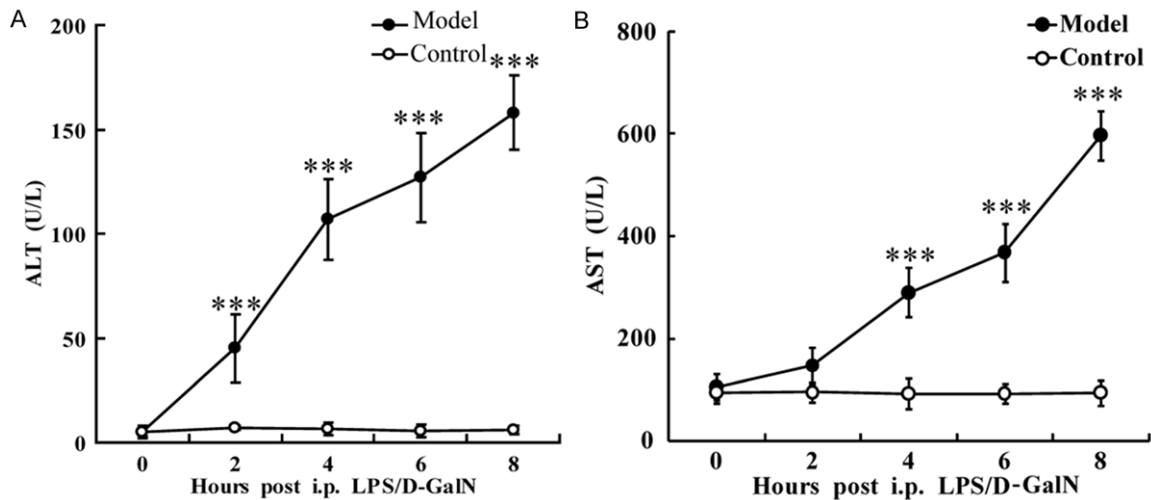

Figure 4. Serum levels of ALT and AST in acute lethal liver injury mice. NF-κB-RE-luc (Oslo) luciferase reporter transgenic mice challenged with LPS (500 μg/kg)+D-GalN (800 mg/kg) or with saline as control. Serum was collected at 0, 2, 4, 6, and 8 h after the challenge. Serum levels of ALT and AST were detected with commercial ALT and AST assay kits. Data are expressed as means ± SD (*n*=6) of three independent experiments. **P<0.01, ***P<0.001.

was performed using SPSS15.0. A level of *P*<0.05 was considered statistically significant.

Results

Establishment of a mouse model for acute lethal liver injury

The results show that the survival of mice with acute lethal liver injury induced by *i.p.* delivery of a high dose of LPS (500 μg/kg) plus D-GalN (800 mg/kg) was 8-10 h. In contrast, the lifespans of mice with acute lethal liver injury

induced by *i.p.* injections of lower doses of LPS (300 or 100 μg/kg) plus D-GalN (800 mg/kg) were significantly longer from 10 h to 42 or 72 h, respectively (*P*<0.001). None of the mice in the control group died (**Figure 1A**).

In addition, the fluorescence intensity of NF-κB activity in acute lethal liver injury mice increased in a dose-dependent manner. The differences in the fluorescence intensity of NF-κB activity between the LPS (100 μg/kg) plus D-GalN (800 mg/kg) group, the 300 μg/kg LPS plus D-GalN (800 mg/kg), and 500 μg/kg LPS plus D-GalN
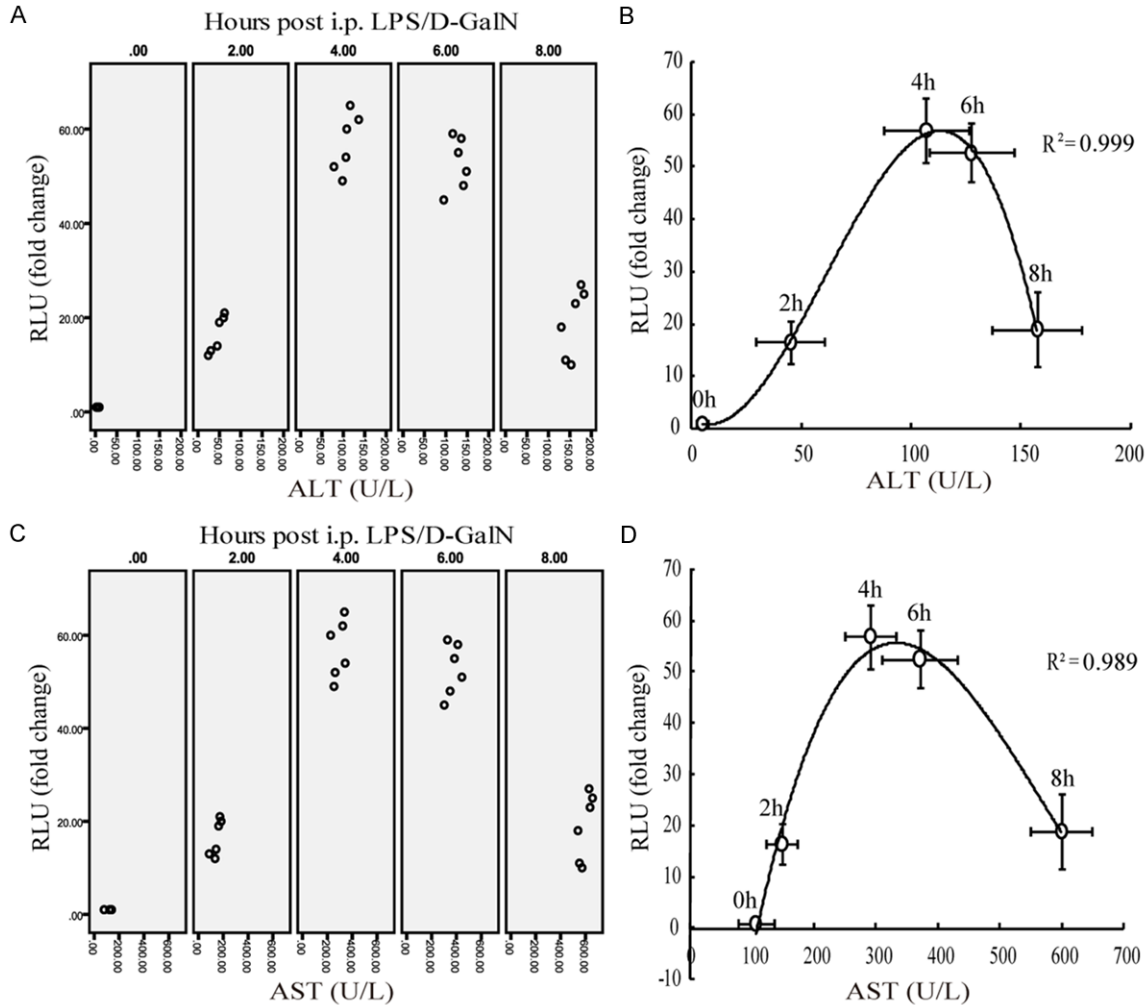
Figure 5. Correlation analysis the relationship between NF-κB activity and serum levels of ALT and AST in acute lethal liver injury mice. Data were collected as described in **Figures 2** and **4**. Correlation analyses of the relationship between the data were made by scatter plot using SPSS 15.0 and polynomial curve fitting (a third order) with Microsoft Excel.

(800 mg/kg) groups (**Figure 1B**) were all significant (*P<0.05*).

In vivo imaging detection of NF-κB activity in acute lethal liver injury mice

The NF-κB activity detected at 0, 2, 4, 6, and 8 h after the acute lethal liver injury model was induced was primarily in the liver and showed a peak at approximately 4 h. The NF-κB activity in the control group was minimal and significantly lower compared with all acute lethal liver injury model groups (*P<0.001*; **Figure 2**).

Serum levels of inflammatory cytokines

As shown in **Figure 3**, the serum levels of MCP-1, IL-6, and TNF-α were significantly increased

in the acute lethal liver injury model group compared with the control group (all comparisons, *P<0.001*). These levels peaked at 2 to 4 h, and then gradually decreasing post-*i.p.* injection of LPS (500 μg/kg) plus D-GalN (800 mg/kg).

Serum levels of ALT and AST and their relationship with NF-κB activity

Serum levels of ALT and AST rapidly increased in the acute lethal liver injury model group over an 8-h period. This increase was significantly higher than the control group (all comparisons except AST at 2 h, *P<0.001*; **Figure 4**).

Analysis of the data by scatter plot (**Figure 5A** and **5C**) and third-order polynomial curve fitting
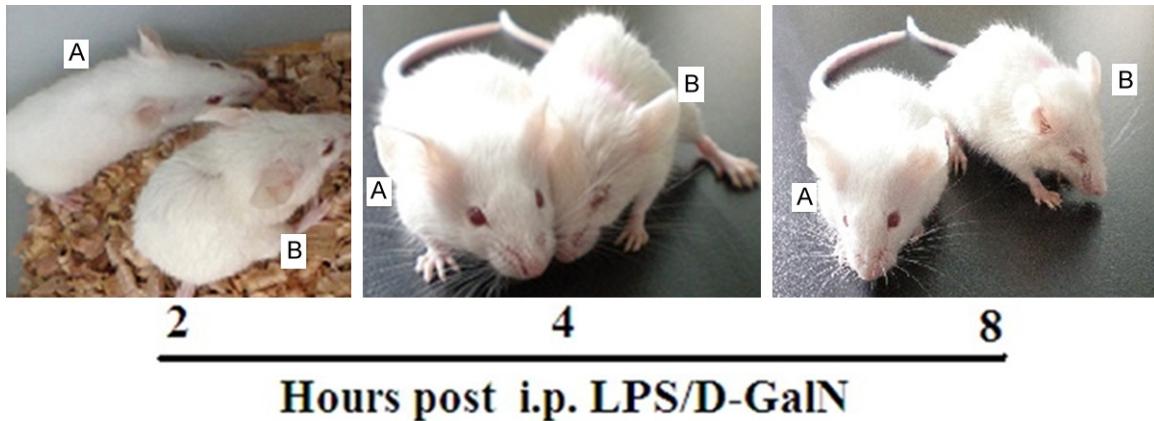
Hours post i.p. LPS/D-GalN

Figure 6. Appearance of acute lethal liver injury mice. Images were taken at 2, 4, and 8 h after NF-κB-RE-luc (Oslo) luciferase reporter transgenic mice were challenged with LPS (500 μg/kg)+D-GalN (800 mg/kg) or with saline as control. A. Control mice. B. Acute lethal liver injury mice.

8h post i.p. LPS/D-GalN

Figure 7. Appearance of livers of acute lethal liver injury mice. Images were taken at 8 h after NF-κB-RE-luc (Oslo) luciferase reporter transgenic mice were challenged with LPS (500 μg/kg)+D-GalN (800 mg/kg) or with saline as control.

(**Figure 5B** and **5D**) showed that NF-κB activity was significantly correlated with serum levels of ALT (R=0.999, *P*<0.01) and AST (R=0.994, *P*<0.01) in the acute lethal liver injury model group.

Changes in liver histology

In contrast with the control mice, acute lethal liver injury model mice showed signs of poor health (dyspnea, delayed corneal reflex, etc.) and aggregation behavior (**Figure 6**). The acute lethal liver injury model mice livers displayed marked congestion and hemorrhage (**Figure 7**). The H&E histological staining showed the destruction of the lobular structure and severe hepatocyte necrosis and hemorrhage, unlike in the control mice (**Figure 8**).

Discussion

A novel LPS/D-GalN-induced, acute lethal liver injury model with visualization of NF-κB activity was established in NF-κB transgenic mice in this study. This model can be used to develop new therapeutic strategies for treating severe acute liver injury complicated by endotoxemia or septicemia.

A previously reported light-producing transgenic animal (LPTA®) model, [NF-κB-RE-luc (Oslo); produced in Dr. Rune Blomhoff's laboratory] includes three response elements (RE) that bind to NF-κB. In this model, forward and reverse primers were used to proliferate 1 KB of the luciferase (luc)-specific genes for genotype analyses. The basic expression sites are located in the cervical lymph nodes, thymus, and Peyer's patches (abdomen). The reporter gene is expressed during inflammation when the transgenic mice are stimulated with LPS and TNF. This model provides the tools to quickly investigate the regulation of NF-κB gene transcription and to develop therapies against inflammation and cancer [4, 20, 21]. A well-known fluorescence imaging system for living specimens is composed of a sensitive charge-coupled device (CCD) camera, analytical software, luciferin, and a luciferase reporter gene. The system has been extensively utilized in the life sciences, medical research, and drug development fields because of its simple operation, intuitive results, and high sensitivity [22, 23].
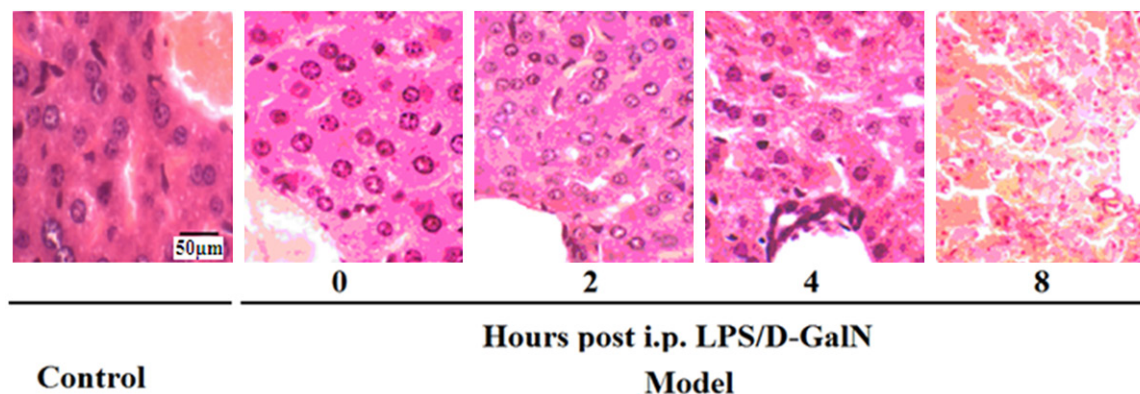
Figure 8. Histology of the livers of acute lethal liver injury mice. NF-κB-RE-luc (Oslo) luciferase reporter transgenic mice challenged with LPS (500 μg/kg)+D-GalN (800 mg/kg) or with saline as control. Livers were collected at 0, 2, 4, and 8 h after challenge and then routinely stained with H&E.

In the present study, a higher dose of LPS/D-GalN than previously used was selected to establish a severe acute lethal liver injury mouse model with a 8-10-h lifespan [11]. Markers related to inflammatory cytokines and liver function, especially the visualization of NF-κB activity, were selected for our model. We found that the fluorescence intensity of NF-κB activity was dependent on the different doses of LPS (100, 300, or 500 μg/kg). IL-6, TNF-α, and MCP-1 were selected as the parameters for the detection and evaluation of severe septic shock [24, 25]. TNF-α is closely related to the survival of mice treated with LPS/D-GalN [4, 5, 26]. In addition, MCP-1 is an important cytokine evaluated in acute lethal inflammation [27]. In the severe acute lethal liver injury mouse model reported here, the serum levels of these cytokines quickly increased, and the liver appearance and function suggested that the model was successfully established.

NF-κB, an early transcription factor that responds primarily to these stimulations, was also investigated. The NF-κB response was consistent with the production of inflammatory cytokines in mice treated with LPS/D-GalN. Moreover, as seen in **Figure 2**, the expression of NF-κB activity was mainly aggregated in liver. Thus, liver injury is the main lethal cause in this model.

LPS, which can induce symptoms similar to endotoxemia or septicemia and manifest as systemic inflammation, was used at a higher dose than usual in this study. However, because this inflammatory reaction is systemic, other organs, including brain, heart, lung, and kidney, can be involved in the death of the model mice. Thus, severe liver injury and systemic inflammation are considered to be the major factors that resulted in the death of the acute lethal liver injury model mice. Moreover, the present study showed that NF-κB activity was significantly correlated with some indicators of liver injury, such as serum levels of ALT and AST.

In summary, this study established an acute lethal liver injury model with a lifespan of 8-10 h. The model, with visualization of NF-κB activity, was induced by using a high dose of LPS/D-GalNin NF-κB transgenic mice. Severe liver injury and systemic inflammation are considered to be the major factors that resulted in the death of the model mice. This novel model will provide a new technology for translational research to develop new therapeutic strategies for treating severe acute liver injury complicated by endotoxemia or septicemia.

Disclosure of conflict of interest

None.

Authors' contribution

All authors have contributed to, read and approved the final manuscript for submission.

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