Published in final edited form as:

J Surg Res. 2006 August ; 134(2): 307–314. doi:10.1016/j.jss.2005.11.588.

TIMP-1/MMP-9 Imbalance in Brain Edema in Rats With Fulminant Hepatic Failure¹

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Abstract

Background—Fulminant hepatic failure (FHF) is a devastating disease. When coma sets in, brain edema develops, changing FHF into a lethal condition. Liver transplantation is the definitive treatment. However, a third of these patients die as the result of brain edema before a donor becomes available. Tissue inhibitor of matrix metalloproteinase (MMP), or TIMP, and MMP-9 are implicated in ischemic brain edema. We thus hypothesized that an imbalance in TIMP-1/MMP-9 relationship plays a role in the development of increased brain extravasation and edema in FHF.

Materials and methods—FHF was induced with a single intraperitoneal injection of D-galactosamine (250 mg/kg). Control rats received saline. GM6001, a synthetic MMP inhibitor, was administered (30 mg/kg) every 12 h for 3 doses starting at 12 h after D-galactosamine injection. MMP-9 was assayed with standard gelatin zymography. Brain extravasation, a measurement of the blood–brain barrier permeability, was determined with Evans blue. Brain edema was determined using specific gravity method.

Results—The active MMP-9 in the systemic circulation was significantly increased in the comatose FHF as compared to the precoma FHF and control animals (6.5 ± 0.7 versus 4.6 ± 0.4 versus 2.6 ± 0.5 pg/ μ g, respectively; $P < 0.05$). Conversely, TIMP-1 was steadily decreased in precoma and coma FHF rats by 35% and 45%, respectively. Blocking MMP-9 activity with GM6001 significantly attenuated brain extravasation and edema in rats with FHF.

Conclusions—Our study strongly supports that the perturbation of decreased TIMP-1 and increased MMP-9 contributes to the pathogenesis of brain edema in FHF. Our findings present a potential therapeutic approach to effectively increase the window of opportunity for life-saving liver transplantation.

Keywords

acute liver failure; brain edema; BBB permeability; TIMP-1; gelatinases

INTRODUCTION

Matrix metalloproteinases (MMPs) collectively are responsible for the regulation of the extracellular matrix in various physiological and pathologic processes [1,2]. Like other MMPs,

¹Supported by a grant from National Institute of Health DK064361 (to J.H.N.) and Deason Foundation.

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MMP-9 and -2 (also known as gelatinases B and A, respectively) are controlled at three levels: 1) transcription and translation in response to cytokines and extracellular stimuli; 2) activation of the proform, proMMP-2 and proMMP-9; 3) extracellular inhibition by the tissue inhibitors of matrix metalloproteinases (TIMPs). In a normal state, TIMP-1 and TIMP-2 strongly bind to proMMP-9 and proMMP-2 as well as to the active MMP-9 and MMP-2 to tightly regulate the MMP activity [1–3]. The presence of active MMP-9 usually indicates ongoing remodeling and potential injury to surrounding organs [1,3].

Early work by Rosenberg and colleagues [4] demonstrated the role of MMP-2 and TIMP-2 in regulation of blood–brain barrier (BBB) permeability in brain ischemic injury. Recently, MMP-9 has been implicated in the pathogenesis of brain edema associated with ischemic and traumatic brain injuries [5–9]. When MMP-9 knockout mice were subjected to cerebral ischemia or trauma, the brain extravasation was significantly attenuated and the neurological functions were protected. Similar protective effects were demonstrated in rats with focal cerebral ischemia when MMP-9 was inhibited using the prototype MMP inhibitor BB-94 [10], or a MMP-9 monoclonal antibody [7]. On the other hand, MMP-2 gene deletion failed to have a protective effect against cerebral ischemic injury [11]. Collectively, these studies suggest that MMP-9 mediates brain extravasation in ischemic brain injury.

Fulminant hepatic failure (FHF) is a devastating liver disease [12]. Brain edema has long been recognized as a major complication of FHF [13,14] and remains to be a major determinant of outcome in FHF [15–17]. In the early stages of FHF, patients may spontaneously recover. However, when coma sets in, brain edema develops, changing a serious condition into a lethal disease [18,19]. Fundamentally, brain edema in FHF results from an increased brain extravasation of water and small molecules [16,20,21]. Although cytotoxic mechanisms have been widely studied, the mechanisms responsible for brain extravasation in FHF remain incompletely understood.

Taken together, we hypothesized that an imbalance in TIMP-1 and MMP-9 regulation contributes to the development of brain extravasation and edema in FHF. In this study, we will show the perturbation in TIMP-1/MMP-9 equilibrium involves a steady decrease of TIMP-1 levels coupled with significant increase in the active MMP-9 in the systemic circulation of rats with experimentally-induced FHF. Inhibiting the active MMP-9 with a synthetic MMP inhibitor GM6001 reduces brain extravasations and edema in the FHF rats.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats, 200 to 250 g of body weight, were purchased from Jackson Laboratory (Bar Harbor, ME). Rats were housed in a conventional mouse room in 12-h light/dark cycles and were provided food and water *ad libitum*. The use of animals was institutionally approved in accordance with The National Institute of Health Guide for the Care and Use of Laboratory Animals.

FHF Model in Rats

The experimental model of FHF that is induced by D-galactosamine (Gal) in rats resembles the disease in humans [22]. FHF was induced with a single intraperitoneal injection of 2.5 g/kg of Gal (CalBiochem) in normal saline. The pH was adjusted to 6.8 with concentrated sodium hydroxide and filtered through a 20- μ m membrane. Control rats were injected with the equivalent amount of normal saline. The rats were observed as they progressed through the clinical stages of hepatic encephalopathy as previously described [22,23]. In brief, in stage I, the Gal-treated rats were almost normal. They had some raised hairs, some decreased activity,

and minimally arched spine. In stage II, the Gal-treated rats showed more decreased activity, more raised body hair, and were lethargic. The rats in stage III had minimal activity and slept most of the time. The righting and pinching reflexes as well as the corneal reflexes were all intact. When the rats started losing the righting and pinching reflexes of the extremities, they entered stage IV of encephalopathy. The rats in stage IV maintained the corneal reflexes. Once the corneal reflexes were lost, indicating brain herniation, death of the animal ensued shortly [24,25]. In this study, precoma indicated stages I and II of encephalopathy. Stages III and IV were referred to as comatose. Serum samples were obtained immediately from sacrificed animals by cardiac puncture. Alanine aminotransferase (ALT) activities were determined in serum using a spectrophotometric assay kit (Biotron Diagnostics, CA). Liver and brain tissues were harvested and preserved in 10% buffered formaldehyde. The tissues were processed for hematoxylin & eosin.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) Gelatin Zymography

This assay allows the detection of both gelatinases, MMP-9 and MMP-2, and their respective proforms, proMMP-9 and proMMP-2 [26,27]. Twenty micrograms of serum protein were electrophoresed at 4°C in 10% SDS-PAGE containing 1 mg/mL of gelatin (Bio-Rad, Hercules, CA). Two nanograms of a mixture containing proMMP-9, MMP-9, proMMP-2, and MMP-2 (Chemicon) were used as standards in each gel. The gels were washed with renaturation buffer containing 2.5% Triton X-100 and then incubated in the development buffer at 37°C for 40 h. Gels were fixed and stained with 0.5% Coomassie Blue R-250 for 2 h. The gelatinase activity was visualized as clear bands against a dark-blue background. The gels were scanned using FluorChem and the bands of the standards and samples were quantitated using Image Quant software (Molecular Dynamics, Hayward, CA).

Measurements of TIMP-1 and -2 Using Reverse Gelatin Zymography

In reverse zymography, pure mixture of MMP-2 and MMP-9 were incorporated within the gel polymerization to allow the detection of TIMP-1 and -2 [28]. Briefly, a 15% polyacrylamide SDS gel was prepared containing 2.5 mg/mL gelatin, 160 ng/mL MMP-2, and 160 ng/mL MMP-9. A standard 4% stacking gel without gelatinases was used. Forty micrograms of each serum protein were applied. The gel was washed with renaturation buffer to remove the SDS for 2.5 h. Then, it was incubated with gentle agitation in the development buffer at 37°C for 40 h. The gel was fixed for 45 min at room temperature in a solution of 50% methanol and 10% acetic acid and was then stained with 0.5% Coomassie Blue R-250 for 2 h at room temperature. The gel was destained for 2 h. Pure human TIMP-1 and TIMP-2 (EMD Biosciences, San Diego, CA) were used as standards. The blue bands against a clear background indicated the presence of a TIMP.

Evans Blue (EB) Extravasation in Rats With FHF

EB has been widely used to assess brain extravasation caused by altered BBB permeability [9,29]. A 2% (w/v) solution of EB at 4 mL/kg was injected intravenously and allowed to circulate for 30 min. The mice were killed, brains were dissected, and photographs were taken. The brain EB extravasation was quantitated spectrophotometrically [9]. Brains were homogenized by vortexing in 250 µL of phosphate-buffered saline (PBS) for 2 min. Then, 250 µL of 60% trichloroacetic acid were added, and the samples were vortexed for 2 min. After cooling for 30 min, the samples were centrifuged for 5 min at 10,000 g. Absorbance readings were measured at 620 nm. EB extravasation was expressed as ng of EB per milligram of brain tissue.

Fluorescent Microscopy for EB Extravasation

EB can be visualized as bright orange under fluorescent microscopy [30]. To process for fluorescent microscopy, we perfused the brains via ascending aorta with 5 mL of saline at 3 feet of height, followed by 5 mL of 4% paraformaldehyde in 0.1 M PBS at pH 7.4. The brains were removed and kept overnight in the same solution. The brains were immersed in 30% sucrose in 0.1 M PBS for 48 h, then frozen in OCT (Sigma) and stored at -70°C . The EB extravasation was observed in the cryostat sections (20 μm) using a fluorescent microscope (Leica DMLB, Germany) with a N2.1 filter, 100-W mercury lamp, MicroMax monochrome camera, and MCID/M5 (Imaging Research, Ontario, Canada).

Brain Water Determination Using Specific Gravity Method

This method has been widely used to measure brain edema [31–33]. The cerebral cortex and cerebellum were quickly removed and stored on an anhydrous tray at 4°C to be processed within 30 min. The gray matter of the cerebral cortex and the cerebellum were cut into 2-mm slices. Then, 1-mm punch biopsy specimens were obtained. These pieces were carefully placed in a bromobenzene–kerosene density gradient column to measure specific gravity, and the equilibrium position was recorded after 2 min, as previously reported [31,32]. The column was calibrated in advance with six potassium sulfate solutions of different concentrations with known specific gravities. All of the gradients used were linear with correlation coefficients ≥ 0.998 . The conversion from specific gravity to brain water was performed as previously described [33]. Eight measurements were made per cerebral cortex and four measurements per cerebellum.

Administration of GM6001

When the prototype MMP inhibitor BB-94 was given intraperitoneally at 30 mg/kg in mice, it produced a plasma level of 20 ng/mL, or 50 nM [34,35]. In this study, GM6001 (Calbiochem, San Diego, CA) was dissolved in dimethyl sulfoxide and then was supplemented in 0.01% polyoxyethylenesorbitan monooleate (Tween 80) in 0.01 M PBS. GM6001 was administered intraperitoneally at 30 mg/kg of body weight every 12 h for 3 doses [36–39], starting at 12 h after the FHF induction.

Data Analysis

Quantitative data were expressed as mean $\pm$ SEM. Statistical comparisons among the study groups were conducted using analysis of variance followed by *t* test with a Bonferroni adjustment for multiple comparisons. A *P* value of 0.05 or less was considered to be statistically significant.

RESULTS

Increased Brain Extravasation at the Onset of Comatose Stages in Rats With FHF

This experimental FHF in rats bears close resemblance to human FHF [22,40]. The clinical progression of encephalopathy, serum aminotransaminase ALT levels (Table 1), and hepatocellular necrosis (Fig. 1) were similar to a previous report [22]. In normal conditions, an intravenous injection of EB would not stain the brain. Accordingly, the brains from control rats did not show a blue staining. There was minimal staining in precoma FHF rats (not shown), which was consistent with a previous report that did not show increased BBB permeability in precoma FHF animals [41]. In contrast, the brains of comatose FHF rats showed marked staining (Fig. 2A). These findings, consistent with previous reports [22,42,43], showed the increased brain extravasation in the comatose FHF rats.

Decreased TIMP-1 Levels and Increased MMP-9 Activity in Systemic Circulation of Rats With FHF

The serum proMMP-9 was increased in FHF rats. These results were in agreement with previous reports that showed elevations of proMMP-9 in livers and sera of FHF and cirrhotic patients and animals [26,44–47]. However, the active MMP-9 in the systemic circulation was not previously reported. In our study, the active MMP-9 was significantly increased in the comatose FHF as compared with the precoma FHF and control animals (6.5 ± 0.7 versus 4.6 ± 0.4 versus 2.6 ± 0.5 pg/ μ g of serum protein, respectively; $P < 0.05$; Fig. 2A). Although proMMP-2 was elevated in FHF animals, MMP-2 was not observed (not shown).

TIMP-1 levels were found to steadily decrease with increasing stages of FHF progression (Fig. 2B). Compared with the normal levels in control animals, the precoma and comatose FHF rats lost 35% and 45% of their TIMP-1 levels, respectively. However, TIMP-2 was detected minimally and did not appear to change with FHF course (not shown).

Imbalance of proMMP-9 and MMP-9 to TIMP-1 in Rats With FHF

To appreciate the stoichiometric relationship of TIMP-1 to proMMP-9 and MMP-9, the molar values of the precoma and comatose FHF rats were divided against those of the normal control animals and summarized in Fig. 2C. This graph illustrates a deviation from normal levels of proMMP-9, MMP-9, and TIMP-1. With the normal at the unity 1, an increased formation in the systemic circulation was indicated by above 1 and a loss by the decrease below 1. In precoma and comatose FHF rats, proMMP-9 increased by 1.3- and 2.0-fold, and the active MMP-9 by 1.8- and 2.6-fold, respectively. In contrast, TIMP-1 levels steadily decreased in the precoma and comatose FHF rats. The imbalanced ratios between proMMP-9 to TIMP-1 and MMP-9 to TIMP-1 increasingly widened in the precoma and comatose FHF rats.

In Vitro Inhibition of Gelatinase Activities by GM6001

When GM6001 or EDTA was present in the incubation buffer of the gelatin zymogram gel, all gelatinase activities were abolished. At 10 nM, GM6001 inhibited 80% of the gelatinase activities (Fig. 3).

GM6001 Attenuated the Brain EB Extravasation and Brain Water

Previous report has shown that if MMP inhibition is administered either before or within 2 h of the FHF induction, the hepatocellular damage is ameliorated [26]. In our study, GM6001 was administered 12 h after the induction of FHF. At this time period, GM6001 did not alter the progress of hepatic necrosis as shown by histology and ALT (not shown). Rather, GM6001 significantly attenuated the brain EB extravasation by 50% in FHF rats (Fig. 4A). Because the EB extravasation into the brain parenchyma could be visualized with fluorescent light microscopy [30], this method was used to confirm the EB extravasation seen on gross examinations of the brains from study animals. EB extravasation could be observed as a generalized fluorescence within the brain parenchyma of the rats with FHF (Fig. 4B), whereas only baseline fluorescence was seen in the control. Treatment with GM6001 significantly ameliorated the EB extravasation in animals with FHF (Fig. 4B).

The brain water content as a measure of brain edema was determined by the specific gravity method [31–33]. The comatose FHF rats had increased brain water greater than the control rats (80.69 ± 0.15 versus 77.70 ± 0.24 ; $P < 0.001$), which was consistent with a previous report [21]. Treatment with GM6001 significantly reduced the brain water increase by 35% ($P < 0.001$; Fig. 4C).

DISCUSSION

Brain edema has long been recognized in FHF [14]. However, the mechanisms responsible for the development of brain edema in FHF remain incompletely understood. Evidence is abundant that MMP-9 plays an important role in various brain injuries. However, the role of a proteolytic enzyme such as MMP-9 in brain edema associated with FHF has not been explored. We preliminarily observed an increase of the active MMP-9 in the systemic circulation of FHF patients and rats [48,49]. Because TIMP-1 tightly regulates MMP-9 activity, we proposed that the altered balance of TIMP-1 to proMMP-9 and MMP-9 results in a net active circulating MMP-9 in FHF.

In this study, we used the rat model of FHF that resembles the human form of FHF. As shown previously [22,42], the comatose FHF rats had marked brain EB extravasation. However, no extravasation was observed in the normal or precoma FHF rats. The EB extravasation depicts the compromised BBB. The brain water in FHF rats, measured by the specific gravity method, increased significantly. Thus, the increased brain water coupled with the EB extravasation suggest a vasogenic mechanism [50] in the pathogenesis of brain extravasation and edema in FHF [51]. Using this rat model of FHF, we found an increase in the active MMP-9 in the systemic circulation of the comatose FHF animals. Similar to previous reports [26,44–47,52], we observed significant increases of proMMP-9. However, proMMP-9 is physiologically inactive. The presence of the active MMP-9 signifies an ongoing remodeling or potential injury to surrounding organs [1]. In physiological conditions, proMMP-9 is secreted along with TIMP-1. Their 1:1 stoichiometric relationship is tightly controlled for fine regulation of the active MMP-9. Available TIMP-1 also binds any free active MMP-9 to keep its activity in check at all times [3]. In our study, we found a significant perturbation in the relationships of TIMP-1 to proMMP-9 and MMP-9. We observed that proMMP-9 increased steadily whereas the TIMP-1 was suppressed progressively. The gap in the imbalance of proMMP-9 and TIMP-1 relationship further widened as the FHF course progressed from the precoma to comatose stages. As a result, the net active MMP-9 increasingly was formed most notably in the comatose FHF rats. The presence of active MMP-9 in circulation in comatose FHF rats suggests a potential role of MMP-9 in the development of brain extravasation and edema.

To correct the imbalance of TIMP-1 and MMP-9 relationship, one may administer TIMP-1. However, the use of TIMP-1 is limited by its short half-life. The synthetic hydroxamate inhibitor GM6001 is effective against MMP-9, MMP-2, and other MMPs [53]. GM6001 and its relatives effectively protect against MMP-9-mediated brain injuries in brain ischemia [10], cold injury [54], and experimental encephalitis [36]. *In vitro*, GM6001 at 10 nM concentration inhibits 80% of the MMP-9 activity when using the gelatin zymography assay. Treatment with GM6001 effectively attenuates the brain extravasation in FHF rats. The extravasation is confirmed with gross inspection, spectrophotometric quantitation, and fluorescent light microscopy. Similarly, GM6001 reduced the brain edema, measured by specific gravity method, in rats with FHF. These results suggest that the restoration of MMP-9 regulation effectively reduces the brain extravasation and edema in FHF. Collectively, our results suggest that an imbalance of TIMP-1 and proMMP-9/MMP-9 plays an important role in the pathogenesis of brain edema in FHF.

A perturbation in TIMP and MMP relationship has been implicated in brain injury [55], cancer metastasis and invasion [56], bronchopulmonary dysplasia [57], and acute myocardial injury [58]. There are four TIMPs known to date, namely, TIMP-1, -2, -3, and -4. They share a high sequence homology. Although a TIMP can inhibit any MMP, TIMP-1 and -2 tend to bind to proMMP-9/MMP-9 and proMMP-2/MMP-2 preferentially [3]. TIMP-2 also plays an important role in the activation process of proMMP-2 to the active MMP-2 [5]. When the equilibrium is altered the opposite direction, the overriding TIMP level can result different

influence on an organ. For instance, an increased TIMP-1 formation can lead to fibrosis in the liver [59]. Thus, TIMP/MMP equilibrium is essential for the wellbeing of an organism.

There is a single report on TIMP-1 in patients with FHF [60]. Nakamura *et al.* [60] measured the serum levels of TIMP-1 using enzyme-linked immunosorbent assay and found them to be significantly elevated in patients with FHF. In our study, the reverse zymography measures only the functional levels of TIMP-1 binding to MMP-9 and MMP-2 that are incorporated within the assay gel. Using this functional assay, we have found a marked reduction of TIMP-1 in FHF patients. We believe that the functional assay may reflect a more accurate level of TIMP-1 in systemic circulation. However, the exact dynamics of TIMP-1 in FHF remain to be further investigated.

In conclusion, the homeostatic equilibrium of TIMP-1 to MMP-9 is dysregulated in FHF. The decreased TIMP-1 with resulting increased MMP-9 strongly supports the important role of MMP-9 in the pathogenesis of brain edema in FHF. These findings provide a basis for pharmacologic intervention with a TIMP-1 equivalent or MMP inhibitor in the clinical management of brain edema in FHF.

ACKNOWLEDGMENT

We thank Kathleen Norton for editorial assistance.

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FIG. 1.

Liver histology of rats with FHF. The liver section from the saline control rats is normal. FHF rats from stage I to IV of encephalopathy showed increasing hepatocellular necrosis, parenchymal disruption, and hemorrhage. The extent of hepatocellular necrosis correlated to the serum transaminases and encephalopathy. GM6001 treatment did not alter the hepatocellular injury or ALT levels in FHF rats (not shown).

FIG. 2.

Increased MMP-9 activities accompanied by decreased levels of TIMP-1 in FHF rats. (A) The activities are determined using gelatin zymography. Both proMMP-9 and MMP-9 are increased significantly in sera of FHF rats. The MMP-9 activities are highest in the comatose stages of FHF (stages III and IV). * $P < 0.05$; ** $P < 0.001$. (B) TIMP-1 levels are decreased with increasing stages of FHF. TIMP-1 and -2 are measured with reverse zymography. No TIMP-2 was observed. However, TIMP-1 steadily disappears in rats with FHF. At the comatose stages III and IV, TIMP-1 losses are 30% and 50%, respectively. * $P < 0.001$. (C) The composite graph demonstrates the deviations of proMMP-9, TIMP-1, and MMP-9 from the normal levels. There was a steady increase in proMMP-9 with a parallel decrease in TIMP-1 level. A net result is a robust increase in the active MMP-9 in animals with FHF.

FIG. 3.

GM6001 inhibits MMP-9 activity *in vitro*. MMP-9 and proMMP-9 are measured using gelatin zymography. In the incubating development buffer, GM6001 is added to final concentrations of 0, 10, 50, and 100 nM. GM6001 inhibits more than 80% of the MMP-9 activities.

FIG. 4.

GM6001 attenuates brain EB extravasation and edema in FHF rats. (A) The normal rats have a trace of EB staining confined within the brain vessels. In contrast, rats with FHF show an intense and diffuse EB staining in the parenchyma and vasculature of the brain (FHF + Vehicle). Treatment with GM6001 significantly attenuates the EB extravasations in FHF rats (FHF + GM6001) by 50%. $*P < 0.001$; GM6001, a MMP inhibitor; EB, Evans blue dye; FHF, fulminant hepatic failure; $N = 5$ rats per study group. (B) Fluorescent microscopy demonstrates the EB extravasations. Baseline fluorescence is seen in the brain of normal control rats (Normal control). Marked and generalized intense fluorescence of the extravasated EB is observed in the brain of FHF rats (FHF + Vehicle). GM6001 significantly attenuates the EB fluorescence in FHF animals (FHF + GM6001). In A (top panel), images are captured at 100X magnifications, demonstrating a generalized view of the brains of the study animals. At 400X (B, bottom), individual brain capillaries with immediate surrounding parenchyma were illustrated. GM6001 treatment markedly attenuates the extravasations of EB from the brain capillaries. (C) GM6001 reduces brain edema. Brain water content is determined using specific gravity method. The brain water content increases significantly in the FHF rats (filled columns) above the normal rats (empty column). Treatment with GM6001 significantly attenuates the brain water content in the FHF animals by 37%. $*P < 0.001$.

TABLE 1

Experimental FHF in Rats

Stage of encephalopathy	Time of onset, hrs	Clinical manifestations	ALT, U/L (Mean $\pm$ SEM)
0	0	Normal	31 $\pm$ 0.3
I	22.6 $\pm$ 5.2	Ataxic	2334 $\pm$ 219
II	36.4 $\pm$ 6.7	Lethargic	3177 $\pm$ 134
III	46.4 $\pm$ 4.5	Comatose, arousable, extremity reflexes intact	3479 $\pm$ 200
IV	50.3 $\pm$ 6.6	Comatose, loss of extremity reflexes, corneal reflexes intact	3837 $\pm$ 118
Brain herniation	—	Loss of corneal reflexes	NA

After a single intraperitoneal injection with D-galactosamine, the rats progress through the clinical stages of encephalopathy from I to IV and eventual death. ALT increases with increasing stages of FHF. The clinical stages and ALT levels correlate to the hepatocellular necrosis in liver histology, Figure 1.

FHF, fulminant hepatic failure; ALT, alanine aminotransferase; NA, not applicable.