Thoughts and Progress (Artificial Organs)

Extracorporeal bioartificial liver using the radial-flow bioreactor in treatment of fatal experimental hepatic encephalopathy

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Short title: BAL for hepatic encephalopathy

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Abstract

We aimed to develop an extracorporeal bioartificial liver (BAL) that could prevent death from hepatic encephalopathy in acute hepatic insufficiency. A functional human hepatocellular carcinoma cell line (FLC-4) was cultured in a radial-flow bioreactor. The function of the BAL was tested in mini-pigs with acute hepatic failure induced by α–amanitin and lipopolysaccharide**.** When the BAL system was connected with cultured FLC-4 to three pigs with hepatic dysfunction, all demonstrated electroencephalographic improvement and survived. We noted relatively low plasma concentrations of S-100 β protein, as a marker of astrocytic damage, from pigs with hepatic failure during BAL therapy. BAL therapy can prevent irreversible brain damage from hepatic encephalopathy in experimental acute hepatic failure.

Introduction

A clinically effective bioartificial liver (BAL) requires development of a high-density cell culture module and a highly functioning liver cell line. We sought to develop a high-performance BAL to avoid lethal hepatic encephalopathy in acute hepatic insufficiency and establish the BAL as an extracorporeal circulation therapy able to surpass conventional blood purification procedures. Our extracorporeal BAL support system used a highly functional human hepatocellular carcinoma (HCC) cell line (FLC-4) cultured in a radial-flow bioreactor (RFB) (1, 2). The RFB is packed with cell-adhesion scaffolds in a cylindrical array (Fig. 1A, B). The culture medium or the plasma flows from the periphery of the cylindrical module to the center. One important problem when cells are cultured densely is delivery of sufficient oxygen and nutrients even when these are plentiful at the inflow site. As a result, we could culture cells successfully at a density of 10^8/ml.

We have currently tested the BAL in mini-pigs with acute hepatic failure induced by α–amanitin, a mushroom-derived poison, and lipopolysaccharide (LPS), while monitoring with electroencephalography (EEG) to assess the effectiveness of BAL against hepatic encephalopathy. We measured the plasma levels of S-100 β protein, a marker of damage to astrocytes (3).

Materials and Methods

Mini-pigs and monitoring

Male mini-pigs (CSK-MS) weighing 10 to 15 kg were a generous gift from Chugai Pharmaceutical (Tokyo). Prior to the experiments they were maintained for 1 to 4 weeks at the Laboratory Animal Facilities of the Jikei University School of Medicine, receiving standard chow and water *ad libitum*. The study was approved by the institution's committee concerning animal experimentation.

Acute hepatic failure model

During inhalation anesthesia with 3 to 4% isoflurane, 0.05 mg/kg of α-amanitin (Calbiochem, Darmstadt, Germany) and 1 μg/kg of LPS (Sigma, St.Louis, MO) dissolved in 10 ml of saline was administered via the splenic vein. Fifty percent glucose solution and 7% sodium bicarbonate were injected as required during the monitoring of venous blood glucose and arterial blood acid-base parameters.

BAL using the radial flow bioreactor (RFB)

The RFB (Biott, Tokyo, Japan) is a cell-filling type bioreactor of 15 ml capacity in which a cylindrical module is filled up with porous hydroxyapatite beads (PENTAX, Tokyo, Japan) with a diameter of approximately 1 mm (1) (Fig.1A,B,C). The culture system consists of the RFB, a reservoir adjusting culture fluid, a circulation pump and an automatic controller to adjust the dissolved oxygen content and the pH of the culture fluid. We injected 10^8 of FLC-4 cells, a human hepatocellular carcinoma (HCC) cell line, into the reservoir, which contained ASF 104 culture medium (Ajinomoto, Tokyo) with 2% fetal bovine serum (FBS) and set the circulated pump at 10 ml/min for seeding and attaching cells into the porous hydroxyapatite beads in the RFB (Fig.1D). Circulation culture at 25

ml/min was continued for 10 days after adhesion of the cells was confirmed and FLC-4 cells grew 10^9 in the RFB. We used the RFB almost completely filled with cells as the BAL.

Extracorporeal circulation using the RFB

Arterial blood was extracted at 20 to 30 ml/min, and plasma was separated at 10 to 15 ml/min by a plasma separator (Plasmaflo, OP-02W; Asahi Kasei Medical, Tokyo) and allowed to circulate through the BAL after passage through an oxygenator (silicone rubber tube module M40-3000; Nagayanagi, Tokyo) (Fig.1E). The entire device was maintained constantly at 37℃. Together with the separated blood cells, the purified plasma was returned to the animal via the cervical vein. The extracorporeal circulation time in this experiment was 4 to 6 hr. Intravenous treatment with 50% glucose and 7% sodium bicarbonate solution continued after the BAL extracorporeal circulation. Heparin was injected as an anticoagulant. At initiation of extracorporeal circulation, an intravenous injection of 1000 units was given. Heparin then was infused into the withdrawn arterial blood at 500 unit /hr.

Enzyme-linked immunosorbent assay (ELISA) for S-100 β in plasma

We measured S-100 β protein in plasma as a systemic marker of damage to brain astrocytes in pigs with acute hepatic failure using an ELISA kit (Yanaihara Institute, Shizuoka, Japan).

Results and Discussion

In the present preclinical study, our ultimate aim was to find a way to prevent or reverse potentially lethal hepatic encephalopathy in acute hepatic failure using BAL as a bridge to either recovery or transplantation. We used an acute hepatic failure model involving a relatively large animal to test the effectiveness of densely cultured FLC-4 cells in a module for extracorporeal circulation. Devising an acute hepatic failure model in a relatively large animal is extremely difficult. We used the method of Takada *et al*., who induced acute hepatic failure by injecting α-amanitin and LPS via the portal vein (4). We first established extracorporeal circulation through a BAL system without FLC-4 cells in two animals (Animal 1 and 2) with α-amanitin/LPS-induced hepatic dysfunction. Animals died respectively 2 hr after initiation of extracorporeal circulation and at a completion point of extracorporeal circulation at 6 hr (Fig. 2A). We thus used the BAL system with FLC-4 cells to treat hepatic dysfunction in three animals (Animal 3, 4, 5), obtaining EEG improvement and survival in all. Figure 2B shows the course of one animal treated by BAL therapy. Even when transaminase and ammonia concentrations in blood were not markedly elevated, many animals that had been injected with α -amanicin and LPS died of hemorrhagic necrosis of the liver with marked cerebral edema. Among the blood tests, the best index of hepatic failure was a decrease in the cholesterol concentration. We therefore administered BAL for 4 to 6 hr, when the EEG showed slowing and plasma cholesterol decreased, about 12-20 hr after toxin administration. Three animals with acute hepatic failure showed considerable normalization of slow-wave activity in the EEG after extracorporeal circulation therapy using FLC-4 cultured in the RFB, with ultimate survival.

The reason for survival of animals with acute hepatic failure treated with the BAL is thought to be the

prevention of rapidly progressive cerebral edema. In plasma from a pig dying from hepatic failure, and a surviving animal just after BAL therapy, we measured the plasma levels of S-100 β protein as a marker of hepatic encephalopathy specifically astrocytic damage (3). S-100 β protein had significantly increased in plasma from animals with acute hepatic failure, especially those that died (Fig.2C). In contrast, we observed that the release of S100- β protein in plasma was inhibited during BAL therapy and animal survived (Fig.2D). This suggested that BAL therapy tended to ameliorate encephalopathy in acute hepatic failure.

Agents potentially responsible for hepatic coma include not only ammonia and manganese compounds, but also an assumed unknown substance with a molecular weight of 5-20 kD (5). Astrocytes form the blood brain barrier (BBB), and sustain nerve cells as they function (6). Increases of the postulated hepatic coma agent in blood induce early functional impairment in astrocytes. However, removal of the unidentified hepatic coma agent(s) is difficult using conventional blood-purifying treatments. We therefore feel an urgent need to develop modalities such as the BAL, in which human liver cells (highly functioning HCC cell lines) are cultured at high density in an RFB. The results of this experiment suggest that BAL can ameliorate hepatic encephalopathy by removal of suspected and/or unknown hepatic coma agents.

Conclusions

We constructed a compact and high functional BAL system using the RFB and a human HCC cell line FLC-4. Large animals with acute hepatic failure induced by α-amanitin and LPS were able to recover from fatal hepatic encephalopathy, and the result was improved by the extracorporeal circulation therapy using this system.

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Figure Legends

Figure 1. The radial-flow bioreactor (RFB) **(A, B)**; The RFB is packed with cell-adhesion scaffolds in a cylindrical array. Culture medium or plasma flows from the periphery of the cylindrical module to the center. SEM observations in FLC-4 cells cultured on hydroxyapatite beads in the RFB **(C, D)**; FLC-4 cells form a single layer on porous hydroxyapatite beads in a cubic array. arrow head; basal side of cells, arrow; apical side of cells, HAB; hydroxy appatite beads, N; nucleus. The extracorporeal BAL system **(E)**; From blood obtained from an artery, and plasma is separated from cells at 10 to 15 ml/min by the plasma separator (PS) and flows into the BAL after taking upoxygen from the oxygeneter (OG). The entire device is maintained consistantly at 37℃. The purified plasma is mixed with blood cells and returned to a vein.

Figure 2. **A:** Time course of biochemical data in control (Animal 2) developing hepatic failure after administration of α-amanitin and LPS. Plasma was perfused through the RFB without FLC-4 cells. Neither control animal (Animal 1 and 2) recovered at any time from fatal hepatic failure. **B:** Time course of biochemical data in an animal (Animals 4) developing acute hepatic failure after administration of α-amanitin and LPS. Plasma was perfused through the RFB containing FLC-4 cells beginning 12 or more hr after toxin administration. All three animals (Animal 4, 5, 6) treated BAL therapy survived. Arrow indicates time of α-amanitin and LPS administration. Black bar indicates extracorporeal BAL perfusion without cells. AST, aspartate amino transferase;

ALT, alanine amino transferase; LDH, lactate dehydrogenase; T.Bil, total bililbin; T.Chol, total cholesterol; PT, prothrombin time; **C:** S-100 β protein in plasma from an animal with untreated acute hepatic failure, showing a marked increase that suggested severe astrocytic damage. **D:** This increase is less prominent during BAL extracorporeal circulation.

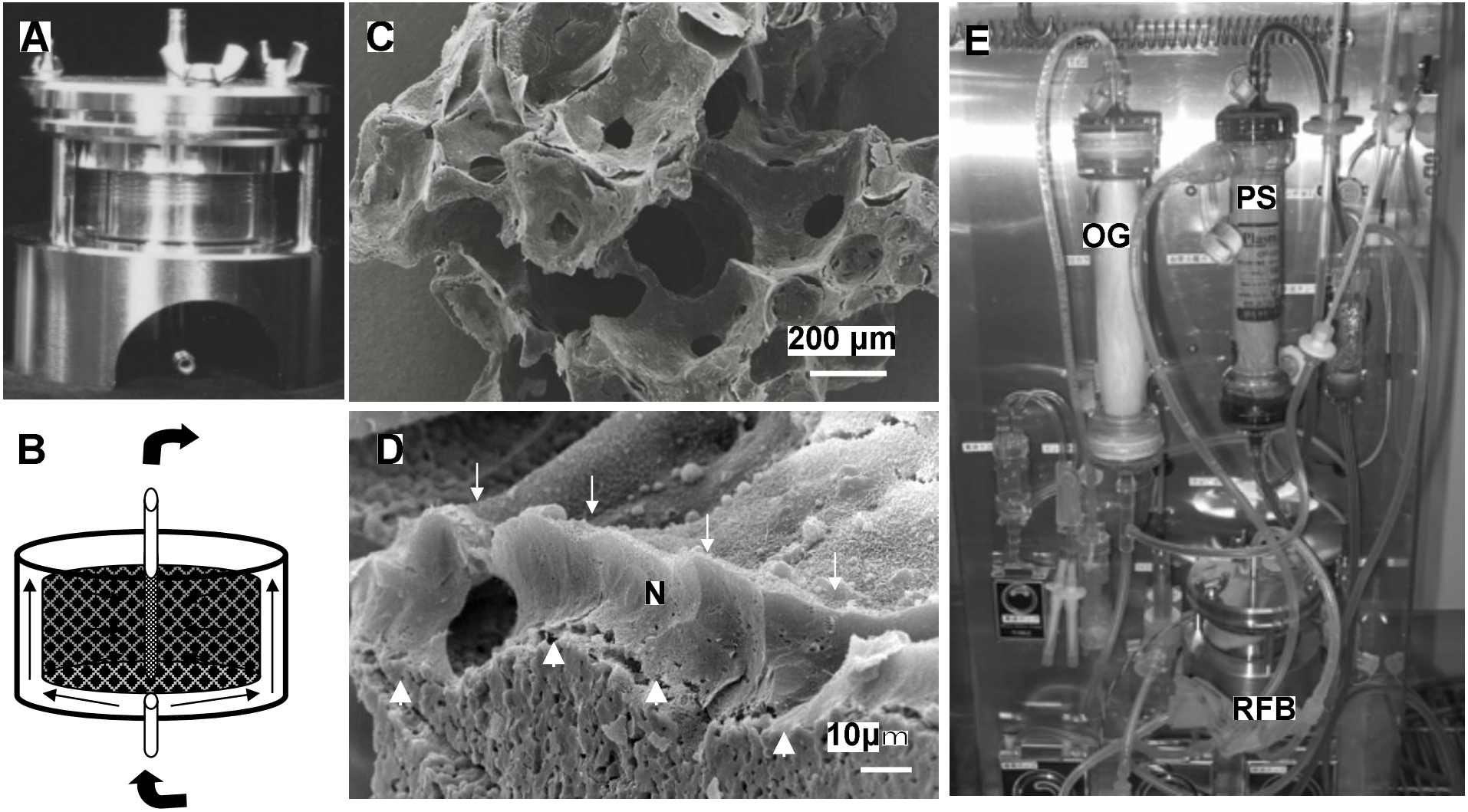

Figure 1

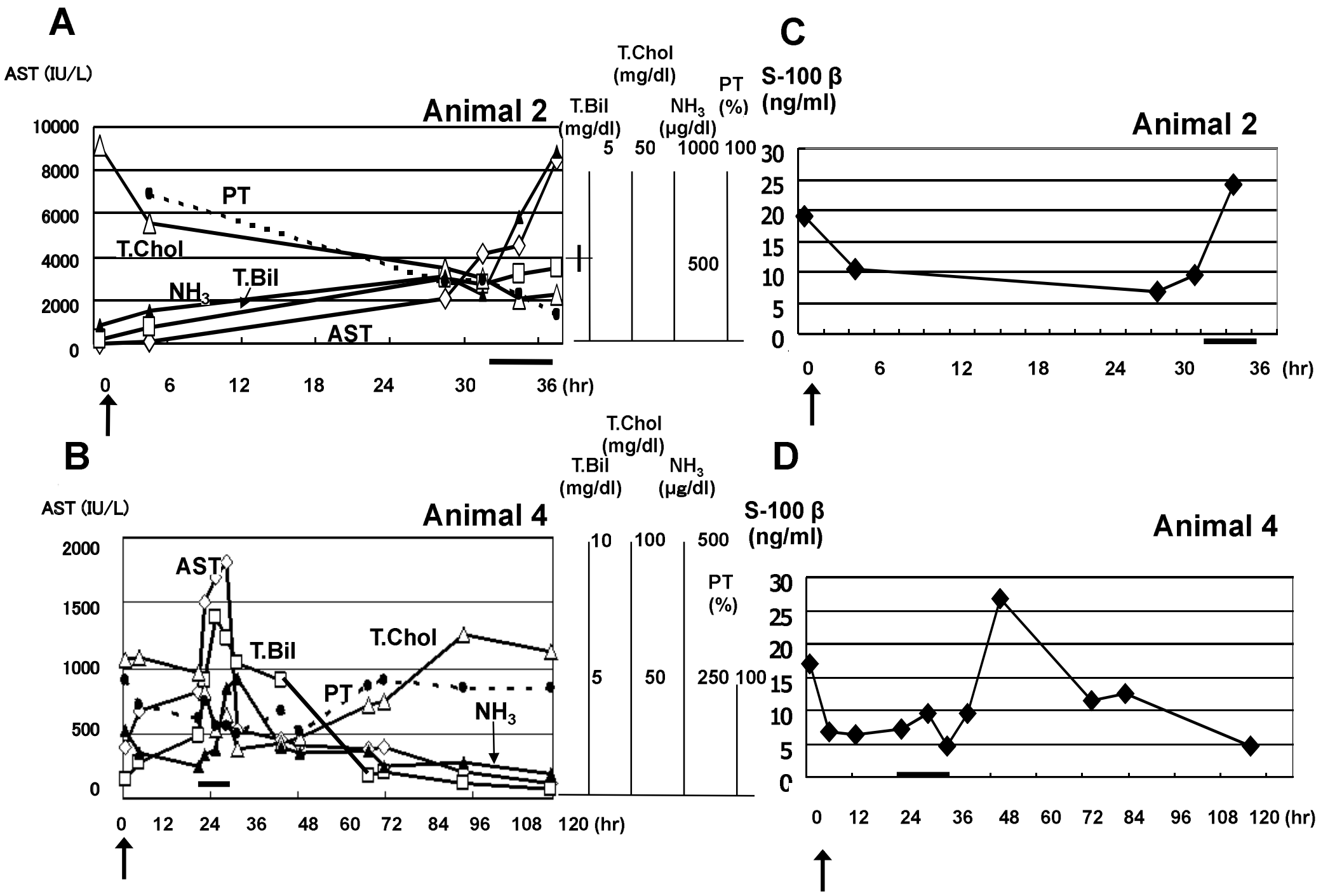
A
AST (IU/L)
Animal 2
T.Chol (mg/dl)
T.Bil (mg/dl)
NH3 (μg/dl)
PT (%)
10000
8000
6000
4000
2000
0
PT
T.Chol
T.Bil
NH3
AST
5
50
1000
100
500
0 6 12 18 24 30 36 (hr)
C
S-100 β (ng/ml)
Animal 2
30
25
20
15
10
5
0
0 6 12 18 24 30 36 (hr)
B
AST (IU/L)
Animal 4
T.Chol (mg/dl)
T.Bil (mg/dl)
NH3 (μg/dl)
2000
1500
1000
500
0
AST
T.Bil
T.Chol
PT
NH3
10
100
500
5
50
PT (%)
250
100
0 12 24 36 48 60 72 84 96 108 120 (hr)
D
S-100 β (ng/ml)
Animal 4
30
25
20
15
10
5
0
0 12 24 36 48 60 72 84 96 108 120 (hr)

Figure 2