Journal of Hepatology 1999; 30: 311–320
Printed in Denmark · All rights reserved
Munksgaard · Copenhagen

Copyright © European Association for the Study of the Liver 1999

Journal of Hepatology
ISSN 0168-8278

Evaluation of a novel bioartificial liver in rats with complete liver ischemia: treatment efficacy and species-specific α-GST detection to monitor hepatocyte viability

Leonard M. Flendrig[1], Robert A. F. M. Chamuleau[1], Martinus A. W. Maas[1], Joost Daalhuisen[1], Brian Hasset[2], Cormac G. Kilty[2], Sean Doyle[2], Nita C. J. J. Ladiges[1], George G. A. Jörning[1], John W. la Soe[1], Dirkje Sommeijer[1] and Anje A. te Velde[1]

[1]*Department of Experimental Internal Medicine, University of Amsterdam, Academic Medical Center, Amsterdam, The Netherlands and* [2]*Biotrin International, Co. Dublin, Ireland*

Background/Aims: **There is an urgent need for an effective bioartificial liver system to bridge patients with fulminant hepatic failure to liver transplantation or to regeneration of their own liver. Recently, we proposed a bioreactor with a novel design for use as a bioartificial liver (BAL). The reactor comprises a spirally wound nonwoven polyester fabric in which hepatocytes are cultured ($40 \cdot 10^6$ cells/ml) as small aggregates and homogeneously distributed oxygenation tubing for decentralized oxygen supply and CO_2 removal. The aims of this study were to evaluate the treatment efficacy of our original porcine hepatocyte-based BAL in rats with fulminant hepatic failure due to liver ischemia (LIS) and to monitor the viability of the porcine hepatocytes in the bioreactor during treatment. The latter aim is novel and was accomplished by applying a new species-specific enzyme immunoassay (EIA) for the determination of porcine alpha-glutathione S-transferase (α-GST), a marker for hepatocellular damage.**

Methods: **Three experimental groups were studied: the first control group (LIS Control, $n=13$) received a glucose infusion only; a second control group (LIS No-Cell-BAL, $n=8$) received BAL treatment without cells; and the treated group (LIS Cell-BAL, $n=8$) was connected to our BAL which had been seeded with $4.4 \cdot 10^8$ viable primary porcine hepatocytes.**

Results/Conclusions: **In contrast to previous comparable studies, BAL treatment significantly improved survival time in recipients with LIS. In addition, the onset of hepatic encephalopathy was significantly delayed and the mean arterial blood pressure significantly improved. Significantly lower levels of ammonia and lactate in the LIS Cell-BAL group indicated that the porcine hepatocytes in the bioreactor were metabolically activity. Low pig α-GST levels suggested that our bioreactor was capable of maintaining hepatocyte viability during treatment. These results provide a rationale for a comparable study in LIS-pigs as a next step towards potential clinical application.**

Key words: **Alpha-GST; Bioartificial liver; Bioreactor; Hepatocytes; Liver support; Oxygenator; Polyester nonwoven; Xenogeneic.**

IN RECENT years many different extracorporeal bioartificial liver designs have been studied (1–21), for potentially bridging the time prior to liver transplantation and treating patients with reversible liver dysfunction (22–24). At present, there are two systems under clinical investigation, both based on porcine hepatocytes (25,26). Short-term application of one of these BAL systems has been associated with some improvements in the condition of patients in fulminant hepatic failure (26), without apparent serious immunological complications (27).

We have devised a novel bioreactor for use as a bioartificial liver (Fig. 1). It comprises a spirally wound,

Received 23 March; revised 12 August; accepted 18 August 1998

Correspondence: Leonard M. Flendrig, Academic Medical Center, Department of Experimental Internal Medicine G2-130, PO Box 22700, 1100 DE Amsterdam, The Netherlands. Tel: +31 20 5665910.
Fax: +31 20 6977192. E-mail: Flendrig@DDS.NL

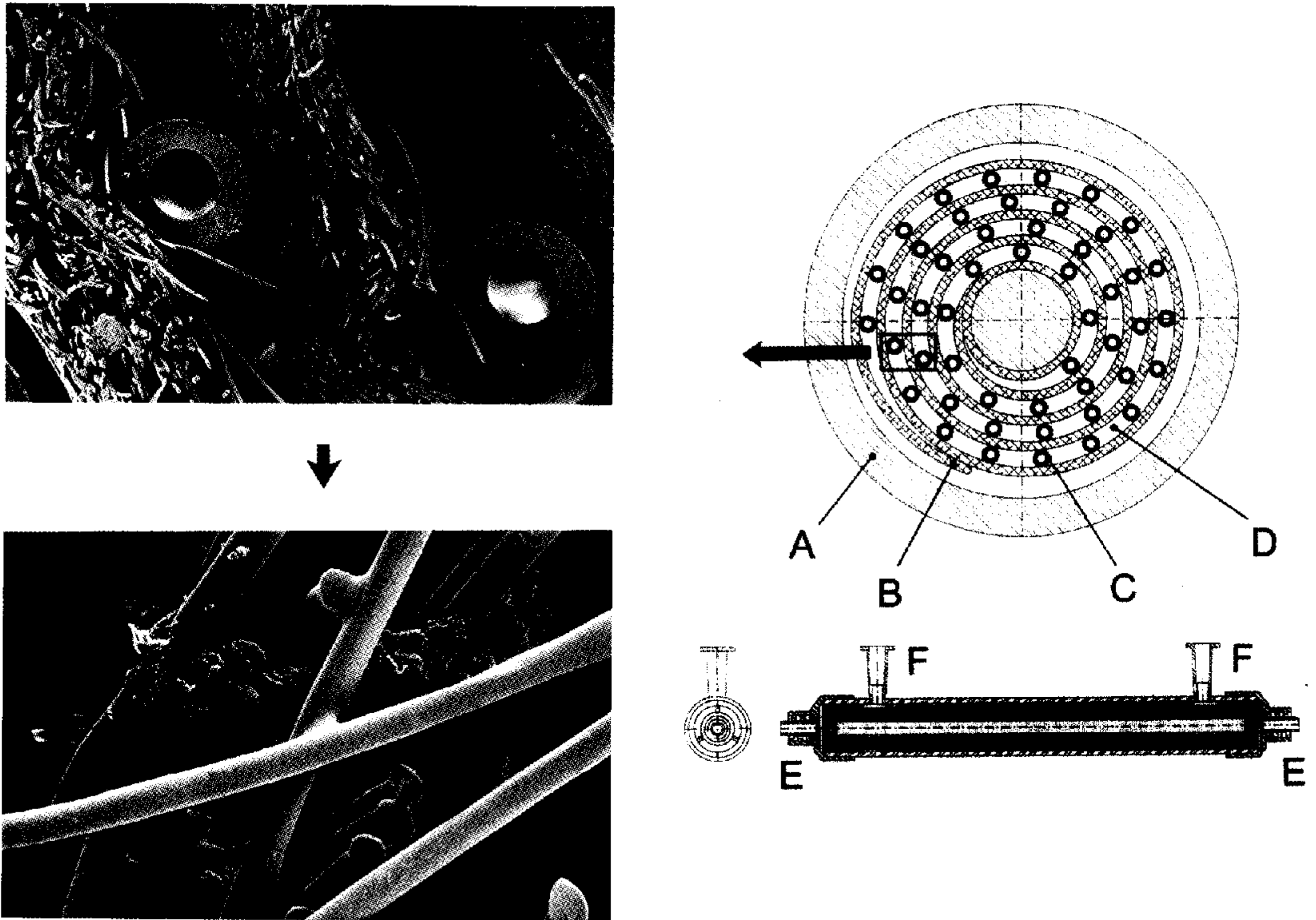

Fig. 1. Schematic drawings of a transverse and longitudinal cross-section of the bioreactor (right), a scanning electron micrograph of a small section of the cell culture compartment (upper left), and a scanning electron micrograph of hepatocyte aggregates attached to a polyester scaffold (lower left, diameter polyester fibers: 13 μm). The system is composed of a polysulfon dialysis housing (A) comprising a three-dimensional nonwoven polyester fabric (B) for high-density hepatocyte culture as small aggregates and hydrophobic polypropylene hollow-fiber membranes (C, external diameter: 630 μm) for oxygen supply and CO_2 removal. The combination of the polyester matrix and the oxygenation tubing creates a third compartment (D). These channels are used to perfuse the plasma of the recipient through the bioreactor. The plasma has direct access to the hepatocytes in the polyester fabric. Plasma is perfused through the bioreactor via the side ports (F). The integrated oxygenator of the bioreactor is connected to the gas supply (5% CO_2 in air) via the endcaps (E). The homogeneous distribution of the oxygenation hollow fibers throughout the bioreactor compartment ensures that every hepatocyte has an oxygen source within its immediate surroundings.

three-dimensional nonwoven polyester fabric in which porcine hepatocytes are cultured at high density ($40 \cdot 10^6$ cells/ml) as small aggregates, and an integrated oxygenator for on-site oxygenation of the cells (28). In contrast to other designs, our bioreactor does not include semipermeable hollow-fiber membranes for cell immobilization, immune isolation, and blood or plasma perfusion (29). Instead, the hepatocytes attached to the polyester fabric function in direct contact with the plasma of the recipient, a situation analogous to that *in vivo*. An immune barrier is accomplished by incorporating two filters into the BAL circuit (Fig. 2). These prevent direct cell-cell interactions between the blood cells of the recipient and the xenogeneic liver cells in the bioreactor (30,31). The hepatocyte aggregates consist only of a few cells and are spaced using the nonwoven polyester fabric. This enables plasma to be perfused over many individual hepatocytes. It results in low diffusion gradients, which are associated with mass transfer in the intact liver. The efficacy of this approach was shown *in vitro*: the urea-synthesising capacity of the porcine hepatocytes in the bioreactor was twice that of hepatocytes in monolayer culture (28). Other functions such as protein synthesis, galactose elimination, and metabolism of amino acids were well maintained over an investigated period of 3 days.

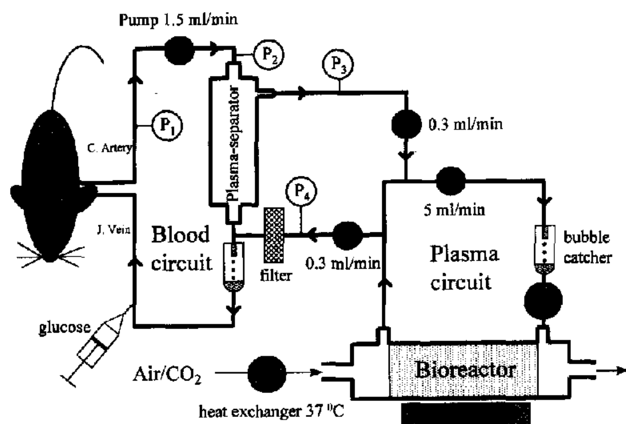

Fig. 2. Schematic representation of the Bioartificial Liver (BAL) support system. A hollow-fiber plasma separator and a filter in the plasma return line were incorporated to prevent cellular immunological problems. Four pressure transducers were used to monitor; the arterial pressure of the rat (P_1), plugging of the hollow-fiber lumen (P_2), fouling of the hollow-fiber membrane wall (P_3), and fouling of the return filter (P_4). A pressure change recorded simultaneously by P_2, P_3, and P_4 indicates a variation in pressure in the venous blood line.

In addition, lidocaine elimination, which was monitored up to 14 days, did not decrease below 70% of its initial value on day 1. Light, transmission and scanning electron microscopic photographs showed viable, well-differentiated cells (with bile canaliculi, tight junctions, mitochondria, and desmosomes) after 5 days in culture (28).

It is well known that plasma of patients with acute liver failure can be cytotoxic to isolated hepatocytes (32). This implies that the metabolic capacity of the hepatocytes in the bioreactor could be impaired while treating the recipient. To ensure optimal liver support, it is important to know when the bioreactor should be replaced by an unused device. This requires an evaluation of the viability of the hepatocytes at regular intervals during BAL treatment. However, this was not feasible until recently. Morphological examination, which used to be the only method available, gives an indication of the viability of the cells at the end of the experiment only, and even then gives only local information. Common biochemical liver tests are not applicable as they do not discriminate between hepatocyte damage in the recipient and the bioreactor. A solution seems to be emerging in the field of xenogeneic BAL treatment. A range of enzyme immunoassay (EIA) kits has become available that allow the determination of the enzyme alpha-glutathione S-transferase (α-GST) in various species. This enzyme is a more sensitive marker for hepatocellular injury than conventional transaminases, as it is uniformly distributed in the liver in high concentrations, is more readily released in response to injury, and has a short *in vivo* plasma half-life (1 hour) (33,34). These uniquely species-specific assays enable the status of bioreactor-based xenogeneic hepatocytes and recipient hepatocytes to be distinguished.

In the present study we tested the efficacy of our novel bioartificial liver system containing porcine hepatocytes in supporting rats with fatal acute liver failure. Species-specific properties of the α-GST EIA tests were applied to monitor hepatocellular damage during xenogeneic BAL treatment.

Materials and Methods

Porcine hepatocyte isolation

Hepatocytes were harvested from male pigs with a body mass ranging from 11–20 kg, using a simple two-step collagenase (collagenase type P; 0.01% wt/vol, Boehringer Mannheim, Germany) perfusion technique, as earlier discussed (35). The viability of the freshly isolated cells was estimated to be 78±4%, based on the trypan blue exclusion test, and the yield varied from $11 \cdot 10^6$ to $25 \cdot 10^6$ hepatocytes per gram wet liver weight. In previous studies we obtained higher cell viabilities, which is possibly related to the use of different types of collagenase (28,35).

Bioreactor

The newly designed bioreactor (patent pending) consists of two principal components: (i) a spirally wound, nonwoven, hydrophilic polyester matrix (Fibra Cell, Bibby Sterilin Ltd, Stone, Staffordshire, UK, diameter polyester fibers: ~13 μm), i.e. an uncoated sheet that provided a three-dimensional framework for hepatocyte immobilization and aggregation, and (ii) integrated hydrophobic polypropylene hollow-fiber membranes donated by AKZO-NOBEL (Plasmaphan, AKZO-NOBEL, Wuppertal, Germany; external diameter: 630 μm) for on-site oxygenation of the hepatocytes (Fig. 1). These two components are enclosed within a polysulfon dialysis housing (Minifilter, Amicon Ltd, Ireland; ID 1.32 cm, ED 1.7 cm, total length 15.5 cm, volume 11 ml). The outer ends of the oxygenation hollow-fibers are embedded in polyurethane resin (PUR-system 725 A and 725 BF, Morton International, Bremen, Germany), using dialyser potting techniques, and are fitted with gas inlet and outlet endcaps.

Hepatocyte seeding and culture

The extrafiber space of the bioreactor was seeded with $4.4 \cdot 10^8$ viable hepatocytes (~4 g) at a concentration of $4 \cdot 10^7$ cells/ml. This was realized by injecting the cell suspension via the side ports (F in Fig. 1) of the bioreactor housing. The cells were cultured for at least 14 h, as previously described (28). The device was then considered to be ready for use and was integrated into the BAL set-up. The same side ports were used to perfuse the plasma of the rat through the cell space of the bioreactor.

BAL system

Figure 2 represents a schematic drawing of the BAL system. It consisted of a plasma circuit including the bioreactor and a blood circuit. Blood was pumped (1.5 ml/min) from the carotid artery of the rat to a mini hollow-fiber membrane plasma filter (Fresenius SSP1003-P, Germany; number of fibers: 80, membrane surface 90 cm^2, membrane material: polypropylene [AKZO-NOBEL, Wuppertal, Germany], membrane pore size: ~0.5 μm, Fresenius, St. Wendel) for continuous separation of plasma from the blood. As plasma could not be withdrawn from the plasma filter at a higher rate than 0.3 ml/min without inducing hemolysis, a high-flow plasma loop (5 ml/min) was introduced to reduce the formation of substrate and metabolite gradients

inside the bioreactor. After recirculation through the bioreactor, the treated plasma was pumped through an outlet filter (Mediakap 5; membrane material: mixed cellulose acetate/cellulose nitrate, membrane pore size: ~0.2 μm, Microgon, Laguna Hills, CA, USA) to remove possible cell debris. This plasma was then reunited with the blood cells from the plasma filter and given back to the rat via the jugular vein. The BAL-system had a total extracorporeal volume of 25 ml (blood circuit 5 ml, plasma circuit 20 ml). A computerized pressure monitoring system was included to monitor the mean arterial blood pressure of the rat, to check the condition of the two filters regarding membrane fouling, and to detect obstruction of the arterial and venous blood lines and catheters. Additional safety features involved controls for gas flow, temperature, and balanced plasma exchange.

Preparation of the BAL support system

The BAL system was prepared for use by priming the plasma separator and the silicon tubing (Silastic, Dow Corning, USA), and flushing the bioreactor with 200 ml pasteurized human plasma solution (PPS, Central Laboratory of The Netherlands Red Cross Blood Transfusion Service) supplemented with glucose (12 mM), bicarbonate (26.1 mM), essential and non-essential amino acids solution (resp. 10 ml/l no. 11130–036 and 20 ml/l no. 11140–035, BRL Life Technologies Ltd, Paisley, Scotland), ornithine (200 μM, Sigma), glutamine (1200 μM, BDH Laboratory Supplies Ltd.), vitamin solution (10 ml/l, Gibco BRL Life Technologies Ltd, Paisley, Scotland), KCl (4.5 mM), $CaCl_2.H_2O$ (2.5 mM), $MgSO_4.7H_2O$ (1.0 mM), insulin (20 mU/ml, Novo Nordisk, Denmark), dexamethasone (1 μM), heparin (2.8 units/ml, Fragmin, Kabi Pharmacia AB, Sweden) and antibiotic/antimycotic solution (5 ml/l, Gibco BRL Life Technologies Ltd, Paisley, Scotland) with pH corrected to 7.4. This "BAL-medium" was used as a first attempt to realize a replacement for both the recipient's plasma and the hepatocyte culture medium. Especially in a clinical setting, it would be of interest to use such a BAL-medium to flush the bioreactor prior to application, as hepatocyte culture media are not approved for medical use. The culture-medium-like composition of the BAL-medium might help to maintain hepatocyte function and integrity.

Experimental animal model of hepatic failure

Outbred male Wistar rats (275–325 g, Harlan Sprague Dawley, TNO Zeist, The Netherlands, kept on a light cycle: 8 am–8 pm) were used and had free access to standard laboratory chow (RMH 1410, Hope Pharms, The Netherlands) and water *ad libitum*. Animal welfare was in accordance with the institutional guidelines of the University of Amsterdam.

Three days before the experiment, an end-to-side portacaval shunt (PCS) was created (36). At day 0, the hepatic artery and the bile duct were ligated under ether anesthesia. Immediately thereafter, 2 ml of 5% dextrose was given intraperitoneally. Carotid artery and jugular vein catheters were placed (ID 0.5 mm, ED 0.9 mm, material: polyethylene, Braun Medical AG, Melsungen, Germany) for plasmapheresis, dextrose administration, blood sample collection and arterial pressure monitoring. The LIS-rats were heparinized with 16.8 units Fragmin/100 g animal weight (Kabi Pharmacia AB, Sweden). After this procedure the LIS-rats were returned to their cage. No further anesthesia was given, allowing the LIS-rats to wake up and move around freely until the onset of coma. During this period the rats had free access to drinking water.

Animal treatment protocol

Three groups of LIS-rats were studied: the first control group (LIS Control) only received a 20% dextrose infusion (0.2 to 0.7 ml/hour) via the venous blood line to prevent hypoglycemia; a second control group (LIS No-Cell-BAL) was connected to the BAL-system without pig hepatocytes; and the treatment group (LIS Cell-BAL) was connected to the BAL-system with $4.4 \cdot 10^8$ viable pig hepatocytes. No other medications were given.

These three different therapies were initiated 30 min after ligating the hepatic artery and bile duct, and were continued until spontaneous death. Several parameters were assessed. The development of liver-associated coma was determined by clinical grading of encephalopathy. Clinical grading was assessed by level of consciousness, which ranges from grade 0 (normal behavior) to 5 (deep coma). In the present study this parameter was extended by an additional grade 6, corresponding to death. In addition to clinical grading, the survival time of all LIS-rats was registered. The mean arterial blood pressure was measured via the carotid artery catheter. The body temperature was monitored rectally and was maintained between 36°C and 37°C with the aid of a heating lamp.

Arterial blood samples were collected just before inducing liver ischemia and at hourly intervals thereafter for determination of ammonia, glucose, and hematocrit. Blood ammonia was measured by the Blood Ammonia Checker II (Kyoto Daaichi Kagaku Ltd, Japan). Blood glucose was measured using the blood glucose checker Medisense (Medisense Inc., Waltham, MA, USA). In all three experimental groups glucose levels were maintained around 10 mM by dextrose infusion if required. Additionally, in the No-Cell-BAL and Cell-BAL groups, lactate, amino acid, and α-GST levels were determined in samples from the plasma circuit just before and at 2-hourly intervals after connecting LIS-rats to the BAL-systems. In this way no blood cells were lost, which was important as the rats in this study had only about 18 ml of blood in their circulation. Lactate was determined at 340 nm (Cobas Bio, Roche, Switzerland) using an enzymatic test kit (no. 149993, Boehringer Mannheim, Wiesbaden, Germany). Amino acids turnover to calculate the Fischer ratio was measured by a fully automated precolumn derivatization with o-phthaldialdehyde (OPA), followed by high performance liquid chromatography as described previously (37). Rat, porcine, and human α-GST were determined in the plasma circuit of the Cell-BAL group. Rat and human α-GST were determined using species-specific enzyme immunometric assays (respectively Hepkit-Rt and Hepkit-Hm, Biotrin International Ltd, Co. Dublin, Ireland). Porcine α-GST was determined using a pre-production EIA, as the Hepkit-Pc was not commercially available at that time. The tests involve the simultaneous addition to microtiter wells coated with anti-α-GST IgG of sample and horseradish peroxidase-labeled anti-GST IgG conjugate, or biotinylated IgG followed by streptavidin-HRP conjugate. The resultant color intensity is proportional to the amount of α-GST present in the sample. Total assay time depends on the assay in question, and is 2 to 3 h.

Sterilization

All components of the BAL system were sterilized by autoclaving (20 min at 121°C) to maximize biosafety.

Statistics

Statistical analysis of survival time (Fig. 3) was performed using the Kaplan-Meier survival test and the Log-Rank test. Continuous vari-

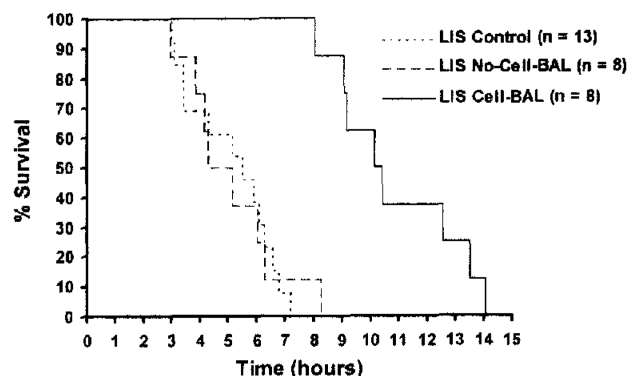

Fig. 3. Percentage of rats surviving as a function of time after inducing liver ischemia (t=0 h). Kaplan-Meier Log-Rank test; $p<0.001$, LIS Cell-BAL versus LIS Control and LIS No-Cell-BAL; $p=0.8931$, LIS Control versus LIS No-Cell-BAL.

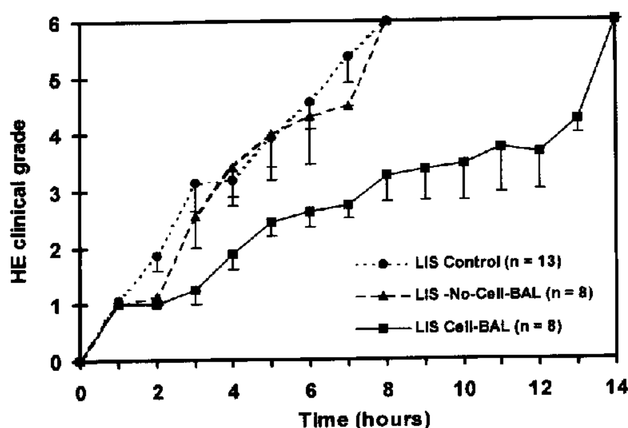

Fig. 4. Development of hepatic encephalopathy in rats after inducing liver ischemia (t=0 h), as by determined by clinical grading. Clinical grading was assessed by level of consciousness, which ranges from grade 0 to grade 5 (0: normal behavior; 1: mild lethargy; 2: decreased motor activity; 3: severe ataxia, no spontaneous righting reflex; 4: no righting reflex to painful stimuli; 5: deep coma, no reaction to painful stimuli). In the present study grade 6 was introduced to indicate death.

ables in Fig. 4 to 7 were assessed by *t*-test for multiple comparisons. The level of statistical significance was set at $p<0.05$. Unless indicated otherwise, data are presented as means±SEM.

Results

In vivo *evaluation of the novel design BAL*

No major technical problems were encountered during the BAL application. After connection of the plasma separation unit, hematocrit decreased on average by 25% due to dilution of the extracorporeal blood circuit with the BAL-medium. No hemolysis was detected throughout the periods that animals were connected to the BAL-system. A small hemorrhage (<1 ml) was observed in the abdominal cavity of some animals. The pressures registered by the pressure transducers P_2, P_3, and P_4 increased slightly during the procedure (10–15 mmHg), indicating moderate fouling of the semi-permeable membranes of the plasma separator and the plasma return filter.

Survival. Analysis of the survival curves (Fig. 3) showed that the LIS-rats in the Control group, the No-Cell-BAL group, and the Cell-BAL group lived, respectively, (mean±sd): 5.4±1.7 h (n=13),. 5.1±1.7 h (n= 8), and 11.0±2.2 h (n=8). There was no statistically significant difference in survival time between the LIS Control group and the LIS No-Cell-BAL group. Life was significantly prolonged in the LIS Cell-BAL group compared to the LIS Control group and the LIS No-Cell-BAL. The immediate cause of death associated with acute liver failure was cardiac arrhythmia and respiratory failure.

Neurological status. In a previous study we have shown that the liver ischemia model of acute liver failure exhibits neurological and behavioral characteristics that enable the degree of hepatic encephalopathy to be readily staged (38). Fig. 4 shows the clinical grade of HE in LIS-rats. The evaluation performed was checked by two trained technicians to limit the degree of subjectivity that is typical for any type of clinical grading. The LIS-rats recovered from ether anesthesia within 1 h after liver ischemia was induced and walked around in their cage. Eventually, all rats developed hepatic encephalopathy (no righting reflex, no response to pain stimuli). The LIS Cell-BAL group showed a statistically significantly delayed deterioration in neurological status compared to the LIS Control group and the LIS No-Cell-BAL group. No statistically significant difference in clinical grading was observed between the LIS Control group and the LIS No-Cell-BAL group.

Hemodynamics. The mean arterial pressure of the LIS rats decreased rapidly after the development of coma grade 4, and reached around 35 mmHg just before death (data not shown). There was considerable variation in the time of onset of coma grade 5. This made a statistical analysis of the arterial pressure data impossible. It was therefore decided to present the mean arterial pressure up to grade 4 (Fig. 5). The LIS-rats receiving the Cell-BAL showed significantly improved hemodynamic stability compared to the LIS Control group. In the Cell-BAL group a decreasing trend in blood pressure was observed beyond 7 h of treatment, but this trend did not reach statistical significance. The mean arterial blood pressure in the LIS No-Cell-BAL group was slightly higher than in the LIS

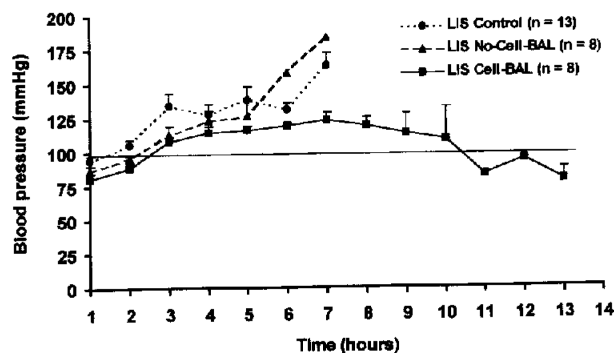

Fig. 5. Shown is the mean arterial blood pressure in three different groups of LIS-rats until onset of coma grade 4 (see results section). The horizontal line represents the mean arterial pressure in healthy normal rats.

Cell-BAL group, but the difference was not statistically significant. Furthermore, no significant differences were observed in the LIS No-Cell-BAL group versus the LIS Control group.

Ammonia metabolism. Data on blood ammonia are shown in Fig. 6. The mean blood ammonia concentration in normal healthy rats (n=30) was 25.8±5 μM. It was 180±21 μM, three days after inducing an end-to-side PCS. Over this period the rats lost 14±3% of their total body weight. In the LIS-rats of the Cell-BAL group the blood ammonia concentration was significantly lower than in the LIS Control group (after t=1 h) and the LIS No-Cell-BAL group (after t=1 h). No statistically significant difference in blood ammonia levels was observed between the LIS Control group and the LIS No-Cell-BAL group.

Lactate metabolism. The lactate levels were monitored by collecting samples from the plasma circuit. The blank samples (Fig. 7) were taken just before connecting the LIS-rats to the BAL. As there was no lactate in the BAL-medium, no lactate was found in the blank samples. Lactate levels rapidly increased in the LIS No-Cell-BAL group after connection to the device. In the LIS Cell-BAL group lactate levels were more stable, and at t=4 h and t=6 h were significantly lower (p<0.003) than those in the LIS No-Cell-BAL group. The lactate determination test was not available at the start of this study. This explains why the number of experiments in the LIS No-Cell-BAL group was five instead of eight.

Amino acid metabolism. HPLC analysis of amino acids from the plasma circuit revealed that the concentration of nearly all amino acids, except arginine, increased after inducing LIS with no significant differences between the experimental groups (data not shown). The Fischer ratio, defined as the ratio of plasma valine, plus leucine, plus isoleucine versus phenylalanine plus tyrosine, decreased in both the LIS No-Cell-BAL group and the Cell-BAL group from approximately 1.45 at 2 h to approximately 0.80 after 6 h of treatment. The ratio did not differ significantly in these two groups (p>0.2).

Hepatocellular integrity. Fig. 8 shows the levels of rat, pig and human α-GST in the plasma circuit of the Cell-BAL group during treatment. Blank samples were collected just before connecting the LIS-rats to the BAL. As a result, no rat α-GST could be detected. The pig α-GST levels in the blank samples were slightly elevated (700±360 μg/l). The rat α-GST levels peaked after 6 h of liver ischemia at 35 000±6000 μg/l and

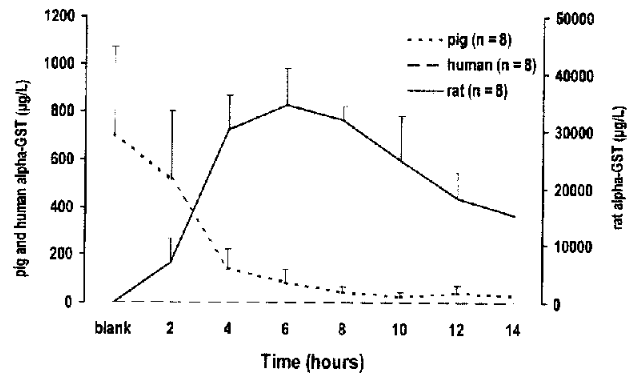

Fig. 8. Rat, pig, and human α-GST levels in the plasma circuit of the Cell-BAL group are shown. The release of this liver enzyme into the circulation is an indicator of hepatocellular damage. The blank sample was collected from the plasma circuit before connecting the LIS-rats to the BAL (see Results section).

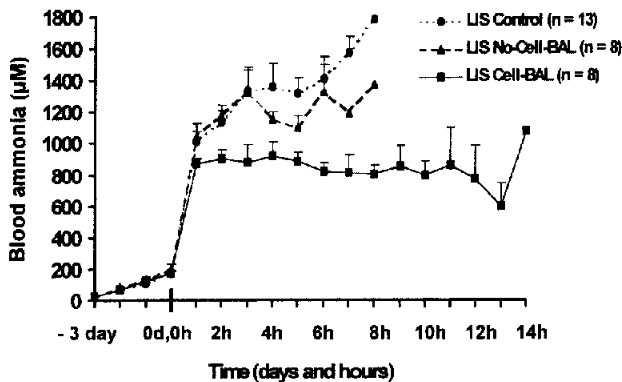

Fig. 6. Representation of the arterial blood ammonia levels in three different groups of LIS-rats. At t=−3 *days an end-to-side portacaval shunt was created, and at day zero liver ischemia was induced (see Results section).*

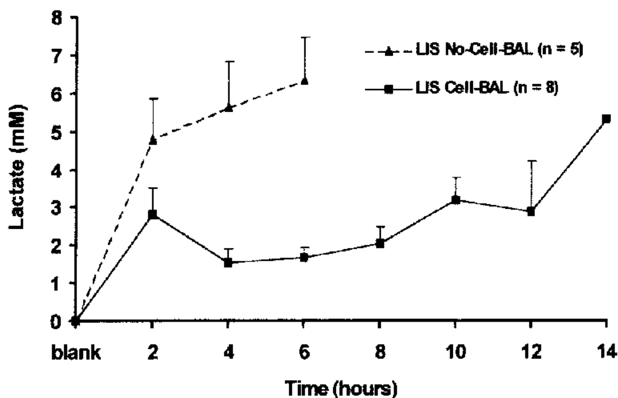

Fig. 7. Lactate levels in the plasma circuit of the LIS No-Cell-BAL group and the LIS Cell-BAL group. The blank sample was collected from the plasma circuit before connecting the LIS-rats to the BAL (see Results section).

rapidly decreased to 15 000 μg/l at 14 h, due to the short half-life of α-GST (60 min). There was no increase in the pig α-GST concentration during BAL treatment. As the BAL-medium contains a human plasma product, it was possible to determine human α-GST and to investigate the cross-reactivity of the human α-GST assay with the rat and pig α-GST. Human α-GST levels remained around 3 μg/l during the experiments and were not influenced by the relatively low pig and very high rat α-GST levels.

Discussion

A true liver support system should be able to prolong life in acute liver failure. This property of a liver support system should be proven *in vivo* in experimental animals before clinical application. Despite numerous studies in the field of extracorporeal BAL support, only a few papers have reported improved survival times (1–5). With the exception of the study of Jauregui et al. (1), the validity of most of these studies may be questioned because of the limited number of animals in the experimental groups. The choice of the model of acute liver failure and the type of anesthesia used are also points of concern (39). A study of D-galactosamine-induced fulminant hepatic failure in unanesthetized dogs (40), revealed better survival than that in an earlier study in anesthetized dogs (41). It was suggested that the difference might be attributable to a possible synergism between halothane anesthesia and D-galactosamine. Furthermore, it was suggested that the halothane-metabolizing capacity of hepatocytes in a BAL could have a protective effect on the recipient liver, which would make previous survival data difficult to interpret (2).

The *in vivo* evaluation of an extracorporeal BAL is a significant challenge. The capacity of our BAL to prolong life was tested by evaluating the system in adequate numbers of unanesthetized animals with irreversible acute liver failure. Our results demonstrated significantly improved survival, delayed onset of HE, lower ammonia and lactate levels, and hemodynamic stability: no hypertension in coma grade 4. The last of these findings might be explained by a beneficial effect of the BAL treatment on the development of brain edema and/or the circulation of vasoconstrictive agents (42). There were no statistically significant differences in survival, onset of coma, hemodynamic stability, and ammonia levels between the two control groups, indicating that there was no device effect and that the BAL-medium was well tolerated by the LIS-rats. Despite a significant amino acid turnover of our system *in vitro* (28), no statistically significant effect was found between LIS-rats connected to a BAL with, and without hepatocytes. Such differences may be masked by the massive release of amino acids from the rat hepatocytes into the circulation after liver devascularization (43).

The cytosolic liver enzyme α-GST is a well-known marker for hepatocyte cell membrane integrity (33,34,44). In addition to its high sensitivity, it is the first marker for hepatocellular injury that can be determined for individual species. Enzyme immunoassays have been developed for rapid (2–3 h) quantitative detection of human, dog, rat, and pig α-GST. These tests may provide a unique tool to investigate the performance of the BAL during *in vivo* experimental and clinical studies, when the cells in the bioreactor are from a xenogeneic source. This is the first study to report on the potential of these assays to discriminate between recipient and bioreactor hepatocyte damage. In addition, the non-invasive character of these tests allowed us to monitor the condition of the hepatocytes in the bioreactor and the liver of the recipient during treatment. To investigate the integrity of the hepatocytes in the present study, the release of rat and pig α-GST into circulation was monitored. The acute character of the liver ischemia model was confirmed by a rapid increase in the rat α-GST concentration after devascularization. The rat α-GST levels peaked at 6 h after inducing liver ischemia, which might suggest that maximum liver damage had occurred at that time. As liver ischemia is a fatal model of acute liver failure, the observed decrease in rat α-GST could not be attributed to cellular recovery but was related to the short half-life of α-GST. Based on these results, it seems likely that the ischemic liver secretes most of the toxic hepatocellular contents into the circulation within 6 h. Accordingly, it was hypothesized that the porcine hepatocytes in the bioreactor would rapidly lose their viability. However, the pig α-GST concentration did not increase during BAL treatment, suggesting that the bioreactor was capable of supporting the porcine hepatocytes over the investigated 14-h period. The pig α-GST levels were slightly elevated before connecting the bioreactor to the LIS-rat. This can be explained by stress induced by the assembly of the BAL and flushing the device with the BAL-medium just before the start of the treatment.

In the development of a bioartificial liver for clinical application the use of hepatocytes from a xenogeneic source is a promising alternative to human hepatocytes because of the shortage of human donor material and the lack of a satisfactory non-tumor human hepatocyte cell line. The pig is considered to be the best candidate for the supply of hepatocytes, since disease-free pig livers can be obtained from specially bred animals and large numbers of cells can be isolated.

A BAL system based on xenogeneic hepatocytes

may present cellular and humoral immunological problems. This could result in either destruction of the hepatocytes in the BAL or in hypersensitivity reactions in the patient receiving BAL treatment (30,31). Proper immunoisolation is therefore essential to ensure safe application of a BAL. Theoretically, this can be achieved by using semi-permeable membranes. The macroporous membranes in the present study were chosen to allow the hepatocytes to function in contact with nearly whole plasma, thereby closely simulating the *in vivo* situation. Cellular immunological complications were circumvented, but no humoral immunological protection was provided. The latter has the following implications for the clinical application of the BAL. Firstly, patients should be screened for human IgM against porcine hepatocytes to prohibit an early malfunction of the bioreactor during BAL treatment (45). Secondly, in a recent study we demonstrated that rats infused with supernatant of cultured pig hepatocytes formed antibodies against pig hepatocyte products (30). As a result, immunological complications may arise in the recipient if the BAL treatment is repeated after 1 week or later. A possible humoral reaction could be serum sickness, a type III hypersensitivity reaction (30,31,46). Others have been using membranes with a molecular weight cut-off of 70 kD (6), and 100 kD (16,47). Although such membranes will prevent anti-hepatocyte-antibodies (>150 kD) and complement factor from entering the bioreactor compartment, hepatocyte-derived products of low and middle molecular weight will still be able to enter the circulation of the patient and induce antibody formation. The liver support capacity of these systems might be impaired, because the hepatocytes are not in contact with all the constituents of whole plasma and only a proportion of the synthesized products will be available to the patient. Recently, an interesting novel membrane has been introduced which allows transport of albumin-bound toxins and free solutes, but prevents the passage of proteins (48). In this way a true immunological barrier can be achieved. Future studies need to prove which of the above-mentioned approaches will be the best compromise. As our filters are located outside the bioreactor, different types of membranes can be applied without having to change the bioreactor design.

In summary, we have developed a novel bioartificial liver system that has been tested in one of the most acute models of liver failure: the liver ischemia model. Besides the absence of liver function, additional stress is introduced by the ischemic hepatocytes secreting toxic cell contents into the circulation. It has been stated that in this model of complete liver failure, BAL support could achieve, at best, a change in the rate of appearance of certain metabolic abnormalities (49). However, in the present study, our BAL was also able to prolong life, delay the onset of neurologic and hemodynamic deterioration significantly, and maintain hepatocyte integrity in the bioreactor. These results were achieved without using a detoxifying resin or charcoal column.

To our knowledge, this study is the first in which such a wide range of improvements has been demonstrated in the LIS model treated with a BAL. As a logical next step, a pre-clinical study in completely devascularized pigs has been initiated, using a scaled-up version of our bioreactor (Microgon, Laguna Hills, CA, USA) that can hold at least $20 \cdot 10^9$ porcine hepatocytes (50). Also, this study is the first using a method to assess the viability of the hepatocytes in the bioreactor during treatment. Because most of the BAL systems currently under investigation are based on porcine hepatocytes, widespread application the novel pig α-GST EIA kit could perhaps increase our understanding of how to improve the efficacy of the BAL treatment and give us an indication of the ability of different BAL designs to support isolated hepatocytes. However, we realise that the use of α-GST EIA requires further validation. An additional study will be necessary to estimate the α-GST content of hepatocytes of different species to obtain the essential quantitative information.

Acknowledgements

We would like to thank Mr. Arie Steenbeek for his skilful technical assistance. We further wish to thank Dr. J. van Marle and Mr. H. van Veen for performing the scanning EM. We are grateful to Dr. E.A. Jones for his critical reading of the manuscript. This research has been made possible by a grant from The Netherlands Digestive Diseases Foundation.

References

1. Jauregui HO, Mullon CJ-P, Trenkler D, Naik S, Santangini H, Press P, et al. *In vivo* evaluation of a hollow fiber liver assist device. Hepatology 1995; 21: 460–9.
2. Sielaff TD, Hu MY, Amiot B, Rollins MD, Rao S, McGuire B, et al. Gel-entrapment bioartificial liver therapy in galactosamine hepatitis. J Surg Res 1995; 59: 179–84.
3. Uchino J, Tsuburya T, Kumagai F, Hase T, Hamada T, Komai T, et al. A hybrid artificial liver composed of multiplated hepatocyte monolayers. ASAIO Transactions 1988; 34: 972–7.
4. Takahashi M, Matsue H, Matsushita M, Sato K, Nishikawa M, Koike M, et al. Does a porcine hepatocyte hybrid artificial liver prolong the survival time of anhepatic rabbits? ASAIO J 1992; 38: M468–72.
5. Kelly JH, Koussayer T, He D, Chong MG, Shang TA, Whisennand HH, et al. Assessment of an extracorporeal liver assist device in anhepatic dogs. Artif Organs 1992; 16: 418–22.
6. Sussman NL, Gislason GT, Conlin CA, Kelly JH. The Hepatix

extracorporeal liver assist device: initial clinical experience. Artif Organs 1994; 18: 390–6.

7. Takeshita K, Ishibashi H, Suzuki M, Yamamoto T, Akaike T, Kodama M. High cell-density culture system of hepatocytes entrapped in a three-dimensional hollow fiber module with collagen gel. Artif Organs 1995; 19: 191–3.
8. Yanagi K, Ookawa K, Mizuno S, Oshima N. Performance of a new hybrid artificial liver support system using hepatocytes entrapped within a hydrogel. Trans ASAIO 1989; 35: 570–2.
9. Miyoshi H, Yanagi K, Fukuda H, Ohshima N. Long-term continuous culture of hepatocytes in a packed-bed reactor utilizing porous resin. Biotech Bioeng 1994; 43: 635–44.
10. Wolf CF, Munkelt BE. Bilirubin conjugation by an artificial liver composed of cultured cells and synthetic capillaries. ASAIO Trans 1975; 21: 16–27.
11. Farghali H, Kameníková L, Martínek J, Lincová D, Hynie S. Preparation of functionally active immobilized and perfused mammalian cells: an example of the hepatocyte bioreactor. Physiol Res 1994; 43: 121–5.
12. Naruse K, Sakai Y, Nagashima I, Jiang GX, Suzuki M, Muto T. Development of a new bioartificial liver module filled with porcine hepatocytes immobilized on non-woven fabric. Int J Artif Organs 1996; 19: 347–52.
13. Sakai Y, Naruse K, Nagashima I, Muto T, Suzuki M. Large-scale preparation and function of porcine hepatocyte spheroids. Int J Artif Organs 1996; 19: 294–301.
14. Bader A, Knop E, Böker K, Frülauf N, Schüttler W, Olderhafer K, et al. A novel bioreactor design for *in vitro* reconstruction of *in vivo* liver characteristics. Artif Organs 1995; 19: 368–74.
15. Kimara K, Gundermann KJ, Lie TS. Hemoperfusion over small liver pieces for liver support. Artif Organs 1980; 4: 297–301.
16. Gerlach JC, Encke J, Hole O, Müller C, Ryan CJ, Neuhaus P. Bioreactor for larger scale hepatocyte *in vitro* perfusion. Transplantation 1994; 58: 984–8.
17. Rozga J, Holzman MD, Ro M-S, Griffin DW, Neuzil DF, Giorgio T, et al. Development of a hybrid bioartificial liver. Ann Surg 1993; 217: 502–11.
18. Joly A, Desjardins J-F, Fremond B, Desille M, Campion J-P, Malledant Y, et al. Survival, proliferation, and functions of porcine hepatocytes encapsulated in coated alginate beads: a step toward reliable bioartificial liver. Transplantation 1997; 63: 795–803.
19. Shnyra A, Bocharov A, Bochkova N, Spirov V. Bioartificial liver using hepatocytes on biosilon microcarriers: treatment of chemically induced acute hepatic failure in rats. Artif Organs 1991; 15: 189–97.
20. Dixit V. Development of a bioartificial liver using isolated hepatocytes. Artif Organs 1994; 18: 371–84.
21. Olumide F, Eliashiv A, Kralios N, Norton L, Eiseman B. Hepatic support with hepatocyte suspensions in a permeable membrane dialyzer. Surgery 1977; 82: 599–606.
22. Matsumura KN, Guevara GR, Huston H, Hamilton WL, Rikimaru M, Yamasaki G, et al. Hybrid bioartificial liver in hepatic failure: preliminary clinical report. Surgery 1987; 101: 99–103.
23. Margulis MS, Erukhimov EA, Andreiman LA, Viksna LM, Temporary organ substitution by hemoperfusion through suspension of active donor hepatocytes in a total complex of intensive therapy in patients with acute hepatic insufficiency. Resuscitation 1989; 18: 85–94.
24. Ellis AJ, Hughes RD, Wendon JA, Dunne J, Langley PG, Kelly JH, et al. Pilot-controlled trial of the extracorporeal liver assist device in acute liver failure. Hepatology 1996; 24: 1446–51.
25. Gerlach JC. Development of a hybrid liver support system: a review. Int J Artif Organs 1996; 19: 645–54.
26. Watanabe FD, Mullon C J-P, Hewitt WR, Arkadopoulos N, Kahaku E, Eguchi S, et al. Clinical experience with a bioartificial liver in the treatment of severe liver failure. Ann Surg 1997; 225: 484–94.
27. Baquerizo A, Mhoyan A, Shirwan H, Swensson J, Busuttil RW, Demetriou AA, et al. Xenobody response of patients with severe acute liver failure exposed to porcine antigens following treatment with a bioartificial liver. Transpl Proc 1997; 29: 964–5.
28. Flendrig LM, La Soe JW, Jörning GG, Steenbeek A, Karlsen OT, Bovée WMMJ, et al. *In vitro* evaluation of a novel bioreactor based on an integral oxygenator and a spirally wound non-woven polyester matrix for hepatocyte culture as small aggregates. J Hepatol 1997; 26: 1379–92.
29. Flendrig LM, te Velde AA, Chamuleau RAFM. Semipermeable hollow-fiber membranes in hepatocyte bioreactors: a prerequisite for a successful bioartificial liver? Artif Organs 1997; 21: 1177–81.
30. te Velde AA, Flendrig LM, Ladiges NCJJ, Chamuleau RAFM. Immunological consequences of the use of xenogeneic hepatocytes in a bioartificial liver for acute hepatic failure. Int J Artif Organs 1997; 20: 229–33.
31. te Velde AA, Flendrig LM, Ladiges NCJJ, Chamuleau RAFM. Possible immunological problems of bioartificial liver support. Int J Artif Organs 1997; 20: 418–21.
32. Hughes RD, Cochrane AMG, Thomson AD, Murray-Lion IM, Williams R. The cytotoxicity of plasma of patients with acute liver failure to isolated rabbit hepatocytes. Br J Exp Path 1976; 57: 348–53.
33. Trull AK, Facey SP, Rees GW, Wight DGD, Noble-Jamieson G, Joughin C, et al. Serum α-glutathione S-transferase: a sensitive marker of hepatocellular damage associated with acute liver allograft rejection. Transplantation 1994; 58: 1345–51.
34. Hughes VF, Trull AK, Gimson A, Friend PJ, Jamieson N, Duncan A, et al. Randomized trial to evaluate the clinical benefits of serum α-glutathione S-transferase concentration monitoring after liver transplantation. Transplantation 1997; 64: 1446–52.
35. te Velde AA, Ladiges NCJJ, Flendrig LM, Chamuleau RAFM. Functional activity of isolated pig hepatocytes attached to different extracellular matrix substrates. Implications for application of pig hepatocytes in a bioartificial liver. J. Hepatol 1995; 23: 184–92.
36. Lee SH, Fischer B. Portacaval shunt in the rat. Surgery 1961; 50: 668–72.
37. van Eijk HMH, van der Heijden MAH, van Berlo CLH, Soeters PB. Fully automated liquid-chromatography determination of amino acids. Clin Chem. 1988; 34: 2510–13.
38. Chamuleau RAFM, Deutz NEP, de Haan JG, van Gool J. Correlation between electroencephalographic and biochemical indices in acute hepatic encephalopathy in rats. J Hepatol 1987; 4: 299–306.
39. Nyberg SL. Galactosamine-induced fulminant hepatic failure [Letter to the Editor]. Hepatology 1997; 26: 1367–49.
40. Diazbuxo JA, Blumenthal S, Hayes D, Gores P, Gordon B. Galactosamine induced fulminant hepatic necrosis in unanesthetized canines. Hepatology 1997; 25: 950–7.
41. Sielaff TD, Hu MY, Rollins MD, Bloomer JR, Amiot B, Hu W-S, et al. An anesthetized model of lethal canine galactosamine fulminant hepatic failure. Hepatology 1995; 21: 796–804.
42. Ellis A, Wendon J. Circulatory, respiratory, cerebral, and renal derangements in acute liver failure: pathophysiology and management. Semin Liver Dis 1996; 16: 379–88.
43. Mazziotti A, Bernardi M, Antonini L, Dioguardi FS, Bellusci R, Papa V, et al. Plasma amino acid patterns in experimental acute liver failure: comparison between hepatectomy and liver devascularization. Surgery 1981; 90: 527–35.
44. Sherman M, Bass NM, Campbell JAH, Kirsch RE. Radioimmunoassay of human ligandin. Hepatology 1983; 3: 162–9.
45. Takahashi M, Ishikura H, Takahashi C, Nakajima Y, Matsushita M, Matsue H, et al. Immunologic considerations in the use of porcine hepatocytes as a hybrid artificial liver. ASAIO J 1993; 39: M242–6.
46. Dixon FJ, Vasquez JJ, Weigle WO, Cochrane CG. Pathogenesis of serum sickness. AMA Arch Pathol 1958; 65: 18–28.
47. Nyberg SL, Platt JL, Shirabe K, Payne WD, Hu W-S, Cerra FB. Immunoprotection of xenocytes in a hollow fiber bioartificial liver. ASAIO J 1992; 38: M463–7.

48. Stange J, Mitzner S. A carrier-mediated transport of toxins in a hybrid membrane. Safety barrier between a patients blood and a bioartificial liver. Int J Artif Organs 1996; 19: 677–91.
49. Rozga J, Morsiani E, Le Page E, Moscioni AD, Giorgio T, Demetriou AA. Isolated hepatocytes in a bioartificial liver: a single group view and experience. Biotech Bioeng 1993; 43: 645–53.
50. Flendrig LM, Di Florio E, Mancini A, Mezza E, Ceriello A, Mazzone M, et al. *In vivo* evaluation of a novel bioartificial liver in a surgical model of acute liver failure in the pig. Preliminary results. In: Crepaldi G, Demetriou AA, Muraca M, editors. Bioartificial Liver Support Systems. The Critical Issues. Rome: CIC Edizioni Internationali; 1997.p. 42–7.