Towards application of a human liver cell line for use in the AMC bioartificial liver
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Evaluation of a novel human fetal liver cell line -cBAL111- in the AMC-BAL in rats with complete liver ischemia

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Abstract

**Background.** Success of clinical application of bioartificial livers (BAL) relies on the development of well differentiated human liver cell lines. A new cell line, entitled cBAL111, has been developed in the Academic Medical Center (AMC) in Amsterdam by immortalization of human fetal hepatocytes by overexpression of human telomerase reverse transcriptase. Previously it has been shown that cBAL111 eliminates ammonia up to 49% of the level of porcine hepatocytes, when cultured in laboratory-scale AMC-BAL bioreactors. In the present study, we evaluated the efficacy of cBAL111 in laboratory-scale AMC-BAL bioreactors in an experimental model of acute liver failure (ALF) using rats with complete liver ischemia (LIS).

**Materials and Methods.** Four groups of LIS rats were studied: 1. a control group for the surgical ALF model, without any connection to a bioreactor (LIS Control; n=4), 2. a second control group connected to an empty bioreactor (Empty BAL; n=4), 3, a third control group connected to a BAL loaded with 450 x 10^6 freshly isolated mature porcine hepatocytes (MPH 450; n=1) and 4. a treatment group connected to a bioreactor with 250 x 10^6 cBAL111 cells (cBAL111 250; n=4). In addition to the *ex vivo* assessment, all cell-based bioreactors were tested in an *in vitro* setting. Functionality of the bioreactors was assessed before and after being connected to the *ex vivo* ALF system.

**Results.** No improvement in the survival time of LIS rats and no differences in blood ammonia levels as well as in the clinical grading of hepatic encephalopathy (HE) were observed after treatment with either cBAL111 bioreactors or empty bioreactors. MPH appeared to have an advantageous effect on survival time, blood ammonia levels as well as the clinical HE score of ALF rats. However, only one experiment using MPH succeeded. After exposure to ALF plasma, ammonia elimination capacity of cBAL111 reversed into ammonia production, but urea production increased, whereas other liver specific functions did not change significantly.

**Conclusion.** In contrast to 450 million freshly isolated porcine hepatocytes, treatment with 250 million of cBAL111 cells in the AMC-BAL did not improve HE and survival time in LIS rats. The detrimental effect of the ALF plasma on the ammonia eliminating capacity of cBAL111 cells in the AMC-BAL may have influenced these outcomes. Extension of the experiment using 450 million cBAL111 cells is needed to draw conclusions on its applicability in BALs. Several aspects of the experimental model of LIS rats as a tool for BAL research need further evaluation.
Introduction

Acute liver failure (ALF) is a serious clinical syndrome that is associated with significant mortality. Orthotopic liver transplantation is still the current treatment of choice for end stage liver disease. However, patients suffering from ALF may benefit from temporary extracorporeal artificial liver support. Bioartificial liver (BAL) support systems are promising as these systems provide detoxification and synthesis function of liver cells.

One of the clinically applied BAL devices, the AMC-BAL, is characterized by direct plasma cell contact, an integrated oxygenation system and a spirally wound polyester matrix, to which small aggregates of porcine hepatocytes are attached. The AMC-BAL, charged with primary mature porcine hepatocytes (MPH), has shown liver specific detoxification and synthesis capacity \textit{in vitro}\textsuperscript{1,2}, significantly improved survival time in small\textsuperscript{3} and large animal models of ALF\textsuperscript{4,5}, and safety in a phase I clinical study.\textsuperscript{6}

Many liver cell types have been used for preclinical and clinical application of BAL devices.\textsuperscript{7} The choice of the optimal cell source for BAL application is, however, particularly defined by the availability, degree of liver specific functions and safety aspects of the cell type used. The most crucial aspect, regardless of the availability and safety, obviously is the potential of liver cells to sort a therapeutic effect in ALF patients.

Recently, a new cell line, named cBAL111, was developed by immortalization of human fetal hepatocytes by overexpression of human telomerase reverse transcriptase (hTERT). This cell line has liver progenitor characteristics \textit{in vitro} and can differentiate into mature hepatocytes \textit{in vivo}.\textsuperscript{8} cBAL111 cultured inside the AMC-BAL displayed hepatic functionality by eliminating ammonia and galactose over a period of 3 days (49\% and 90\% of MPH, respectively). Lidocaine elimination and urea production were nevertheless low when compared to MPH, \textit{i.e.} 0.1\% and 6.0\%, respectively.\textsuperscript{9} Ultimately, the potential of cBAL111 for future BAL application needs to be determined in an animal ALF model.

In the present study, we tested the efficacy of cBAL111 in laboratory-scale AMC-BAL bioreactors in an ALF model using rats subjected to liver ischemia (LIS). This model was adopted from Flendrig \textit{et al.}\textsuperscript{3} who showed a prolonged survival time using MPH. Our main objectives were to evaluate the cBAL111 cells in the AMC-BAL by assessing metabolic function and treatment efficacy in terms of prolonged survival in LIS rats. In addition, the impact of exposure to ALF plasma on cBAL111 bioreactors was tested in an \textit{in vitro} setting before and after BAL treatment.
Materials and Methods

Experimental groups and study design

Four groups of LIS rats were studied: 1. a control group for the surgical ALF model, without any connection to an extracorporeal system (LIS Control; n=4), 2. a second control group connected to an empty bioreactor (Empty BAL; n=4), 3. a third control group connected to a BAL loaded with 450 x 10^6 freshly isolated mature porcine hepatocytes (MPH 450; n=1) and 4. a treatment group connected to a bioreactor with 250 x 10^6 cBAL111 (cBAL111 250; n=4). Figure 1 describes the time-course of the study design. In general, bioreactors were charged with cells at day 1 and tested in vitro at day 2 (pre-test). At day 3, all rats underwent a LIS procedure, followed by the procedure of the group they were assigned to, until death occurred. Bioreactors loaded with cells were then tested in vitro for a second time (post-test). All procedures were conducted in accordance with the institutional guidelines of the Animal Ethical Committee (DEC) of the Academic Medical Center and the Dutch Genetically Modified Organism Organization (GGO).

Monolayer cBAL111 culture and isolation

Large-scale expansion of cBAL111 was performed in Corning® CellSTACK® culture chambers (5-stacked; 3.280 cm² culture area) using DMEM culture medium (Dulbecco’s...
modified Eagle’s medium, BioWhittaker) containing 10% (v/v) heat-inactivated fetal bovine serum (HI-FBS, BioWhittaker), 2 mM L-glutamine (BioWhittaker), 1 μM dexamethason (Centrafarm), 10 μg/mL insulin, 5.5 μg/mL transferrin, 6.7 ng/mL selenium-X (ITS mix, Life Technology), 100 U/mL penicillin, 100 μg/mL streptomycin (penicillin/streptomycin mix, BioWhittaker). Cells were detached with 0.25 % trypsin / 0.03 % EDTA (BioWhittaker), centrifuged at 50x g for 5 min and washed two times with cold DMEM culture medium and one time in cold William’s E (WE) culture medium supplemented with 4% (v/v) HI-FBS, 2mM glutamine, 1 μM dexamethason, 20m U/mL insulin, 2 mM ornithine, 100 μg/mL streptomycin, 100 U/mL penicillin and 0.25 μg/mL amphotericine B. Cell quantity was determined by using a Bürker Bright line cytometer (Optik Labor).

Porcine hepatocyte isolation

MPHs were isolated from livers of female pigs (20-24 kg) by a two-step collagenase perfusion technique according to a modified protocol of Seglen10 as previously described.2 MPHs were suspended and washed in ice-cold WE culture medium, consisting of William’s E medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (BioWhittaker, Verviers, Belgium), 2mM glutamine, 1 μM dexamethason, 20m U/mL insulin, 2 mM ornithine, 100 μg/mL streptomycin, 100 U/mL penicillin and 0.25 μg/mL amphotericine B. Total yield of isolated hepatocytes was estimated by determination of the cell pellet volume after three washing steps in WE culture medium by centrifugation at 50xg for 3 min 11. Viability was determined by trypan blue exclusion test.

Bioreactor culture

General bioreactor configuration has been described in detail by Flendrig et al.1,3 In this study, we used a modified laboratory scale AMC-BAL bioreactor with an internal volume of 10 mL and a double number of gas capillaries. The bioreactors were loaded with 8 ml cell suspensions and placed in a temperature controlled culture cabinet (37°C) and oxygenated with culture gas (95% air / 5% CO₂; flow: 50 mL/min). Bioreactors were subjected to a 340° transverse rotation for 2 hours for MPH bioreactors or 4 hours for cBAL111 bioreactors to ensure optimal cell attachment and an even cell distribution. After this attachment period, dead and unattached cells were removed by flushing 60 mL WE culture medium through the bioreactor at 5 mL/min. Bioreactors were then continuously perfused with 76 mL recirculating WE culture medium at 5 mL/min.

Bioreactor tests

All bioreactors charged with cells were tested 1 day prior to the ex vivo experiment and directly after the ex vivo experiment. The test sequence consisted of an oxygen
consumption test (OCT) and a function test. OCT was used to assess global metabolic activity inside the bioreactor, as described recently. Briefly, oxygen consumption was determined by measuring the decrease in oxygen tension at the medium outflow port during the first 20 min after closure of the oxygen supply to the bioreactor. Function tests were performed by flushing bioreactors with 30 ml of test medium composed of WE culture medium supplemented with 500 μg/ml lidocaine·HCl, 2 mM L-lactate and 5 mM NH₄Cl followed by recirculation of 36 ml test medium for 3 hours at 37°C. Samples (2 ml) were taken at 30, 60, 120 and 180 min and analyzed for concentrations of ammonia, urea, lidocaine, galactose, glucose and lactate as well as activities of aspartate aminotransferase (AST) and lactate dehydrogenase (LDH). Ammonia, lidocaine and galactose clearances,
urea synthesis capacity, AST and LDH release, glucose and lactate consumption and/or production rates were determined by calculating the changes in concentration in test medium per hour per billion loaded cells as described previously.9

**Experimental animal model of hepatic failure**

Male Wistar rats (Han:Wist; 325-350 gr) were kept at a light cycle of 8 AM to 8 PM and had free access to standard laboratory chow and water. At day 0, an end-to-side portocaval shunt (PCS) was created after mid-line laparotomy under inhalation anesthesia with 2-3% isoflurane (O₂/NO₂ 1:1) and analgesia with 0.006 mg/kg buprenorfine s.c.. Before closure of the abdomen, 2.5 ml 5% glucose solution was given i.p., and after awakening, rats had free access to either 5% glucose / 0.9% NaCl solution or normal water. Weight and general welfare were checked daily. At day 3 and under isoflurane anesthesia alone, catheters (0.5 x 0.9 mm; Braun Melsungen AG) were placed in the carotid artery and jugular vein for connection of the rat to the *ex vivo* bioreactor system. After reopening the mid-line laparotomy, the hepatic artery and bile duct were ligated, initiating complete ischemia of the liver (t = 0). Before awakening, 2.5 ml 5% glucose solution i.p. and 16.8 U/100 gr dalteparine i.v. were administered. No further anesthesia was given, allowing the rats to wake up and move around freely until the onset of coma.

**Ex vivo bioreactor support system**

The system (Fig. 2) consisted of three fluid circuits: 1. blood circuit, 2. plasma circuit and 3. bioreactor circuit. Blood was withdrawn from the rat via the carotid artery catheter at 1.5 ml/min and directed through the mini plasmapheresis module (105 mm effective length, 8 mm ID, 80 Plasmaphan capillaries with 0.47 μm max. pore size and total membrane surface of 90 cm²; Membrana GmbH, Wuppertal, Germany) for continuous separation of plasma from blood. Plasma was withdrawn with 0.3 ml/min from the plasmapheresis module and directed to the bioreactor circuit in which it was recirculated at 5 ml/min. Simultaneously, plasma was redrawn from the bioreactor circuit at 0.3 ml/min and directed through a 0.45 μm filter (Millex HV, Millipore Corp, Bedford, USA) to remove possible cellular debris. The filtered and treated plasma was then combined with blood cells from the plasmapheresis module and given back to the rat via the jugular vein at 1.5 ml/min. A pressure monitoring system (HP56S/M1165A multi-channel recorder, Hewlett-Packard, Böblingen, Germany) using four pressure transducers (TruWave, Edwards Lifesciences, Irvine, USA) was included to monitor the mean arterial pressure of the rat and to check pressure changes. These changes indicate possible membrane fouling, i.e. of the plasmapheresis module and the 0.45 μm filter, and any obstructions in the carotid or jugular catheters or in the silicon tubings of the *ex vivo* system. Both the blood circuit and the bioreactor circuit could be operated independently as a stand-alone system.
circuit. The plasma circuit was coupled to the blood circuit. Figure 3 shows an example of an operational *ex vivo* bioreactor system.

![Diagram of a bioreactor system](image)

**Figure 3.** Operational *ex vivo* bioreactor support system. See Appendix 2 for color image.

**Preparation of ex vivo bioreactor support system**

Before priming the *ex vivo* bioreactor system, a plasmapheresis module was hydrophilized by flushing 80 ml isopropanol through the capillaries followed by a rinse of 400 ml 0.9% NaCl. Bioreactors containing cells were then prepared for transfer from the culture cabinet to the *ex vivo* bioreactor system by flushing 100 ml human pasteurized plasma solution (HPPS, Central Laboratory Blood Transfusion Service, the Netherlands) supplemented with 10 mM D-glucose, 0.2 mM L-ornithine.HCl, 4.5 mM KCl, 2.5 mM CaCl$_2$·H$_2$O, 1.0 mM MgSO$_4$·7H$_2$O, 20 mM bicarbonate, 2.8 U/ml dalteparine, 20 mU/ml insulin (Actrapid), 1μM dexamethason, 20 ml/l essential amino acids solution (50x) and 10 ml/l non-essential amino acid solution (100x), 10 ml/l vitamin solution (100x), 100 μg/mL streptomycin, 100 U/mL penicillin and 0.25 μg/mL amphotericine B. All three fluid circuits were primed with supplemented HPPS before the plasmapheresis system and bioreactor were incorporated in the whole system. The blood circuit was primed with blood just prior to the connection of the rat to the system. This blood was obtained by a single heart puncture under 2-3% isoflurane anesthesia of a male Wistar donor rat (Hann:Wist; >350 gr). Blood was collected in an anticoagulant citrate phosphate dextrose solution (CFD; 161 mM D-dextrose monohydrate, 88.7 mM sodium citrate dihydrate,
17 mM citric acid (anhydrous) and 16.1 mM NaH$_2$PO$_4$·H$_2$O and directly used for priming or stored at 4°C for erythrocyte replacements.

**Ex vivo experiment**

Rats were connected to the *ex vivo* bioreactor system within 15 minutes after ligation of the hepatic artery and common bile duct. Rats were able to move freely and were monitored until death. All groups received a 20% glucose infusion via the venous line to maintain blood glucose level at a physiological level (5 - 9 mM; 0.4 to 1.4 ml/hr depending on the activity of the rat). Anticoagulation was realized by continuous infusion of 10 U/hr (0.1 ml/hr) dalteparine via the arterial line. To prevent dehydration, all rats received additional HPPS infusion (0.5 to 1.5 ml/hr depending on the glucose administration). Total infusion was 2.0 ml/hr for all rats. Body temperature was monitored rectally and kept between 36.7 and 37.3 °C with the aid of a heating lamp.

Progression of ALF was monitored via several parameters at hourly intervals. Clinical grading of hepatic encephalopathy (HE) was used to determine the development of liver-associated coma. Grades of consciousness ranged from 0 (normal behavior) to 6 (death) and was performed by two observers (Table 1.). Mean arterial blood pressure was measured via the carotid artery line. Blood samples (0.5 ml) were taken from the arterial line to determine the concentration of ammonia (Blood Ammonia Checker II, Kyoto Daiichi Kagaku Co., Ltd, Japan), glucose (Glucose Checker), hemoglobin (OSM3, Radiometer, Copenhagen), pH, pCO$_2$, pO$_2$ and base excess (ABL505, Radiometer, Copenhagen). Concentration of creatinine, total bilirubin, lactate and aspartate aminotransferase (AST)

<table>
<thead>
<tr>
<th>Grade</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>normal behavior</td>
</tr>
<tr>
<td>1</td>
<td>mild lethargy</td>
</tr>
<tr>
<td>2</td>
<td>slow, decreased motor activity, poor posture control</td>
</tr>
<tr>
<td>3</td>
<td>severe ataxia, no spontaneous righting reflex, diminished response to pain stimulus</td>
</tr>
<tr>
<td>4</td>
<td>no righting reflex on pain stimulus</td>
</tr>
<tr>
<td>5</td>
<td>deep coma, no reaction on pain stimulus</td>
</tr>
<tr>
<td>6</td>
<td>death</td>
</tr>
</tbody>
</table>

*Table 1. Clinical grading of hepatic encephalopathy.*
were determined in plasma by routine laboratory analysis. After taking each sample, 0.5 ml donor blood was given back to prevent excessive loss of erythrocytes by blood sampling.

The rat was disconnected from the *ex vivo* bioreactor system after death occurred and autopsy was performed. To assess the integrity of the LIS procedure, 20 ml of 1:10 trypan blue solution was injected via the carotid artery line. The LIS procedure was considered successful when the liver was not colored blue.

**Statistical analysis**

Repeated measurement rank ANOVA tests were used to compare differences between the four experimental groups. Paired student’s t tests were used to compare differences between pre- and post- *in vitro* tests of cBAL111 bioreactors. SPSS 12.0.1 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Prism version 4.0 (GraphPad Prism Inc, San Diego, CA, USA) was used for graphical presentation of the data. Average values (± standard error) are reported. Significance was reached if *P* < 0.05.

**Results**

**Cell sources**

cBAL111 bioreactors (n=4) were loaded with 250 x 10^6 cells originating from an average of three 5-stack culture chambers with cBAL111 cells grown until confluency. The average yield per 5-stack was 90.7 ± 12.4 x 10^6 cBAL111 cells. One bioreactor was loaded with 450 x 10^6 MPH originating from one liver cell isolate with a viability of 97% and total yield of 13.6 x 10^9 viable cells.

**Ex vivo experiment**

*Excluded ex vivo experiments and animal loss*

The number of experiments, as conducted in this study, was strongly dependent on the availability of the cell source, culture capacity and logistics concerning operating facility program. The consequence of an unsuccessful cBAL111 250 *ex vivo* experiment was a delay of at least 2 weeks, since only in this time-frame a sufficient amount of cBAL111 cells could be obtained for the following experiment as well as for a new batch for further cBAL111 expansion. Nevertheless, between February 2006 and November 2006, we were able to initiate 27 *ex vivo* experiments. The number of *ex vivo* experiments using cell-based bioreactors were divided as follows: 6x 250 million cBAL111, 2x 450 million cBAL111, 3x 250 million MPHs and 5x 450 million MPHs (Table 2).
An overview of the number of surgical operations and initiated \textit{ex vivo} experiments, including the problems and causes of excluded \textit{ex vivo} experiments and animal losses, is given in Table 2. No general cause can be given for the exclusion of experiments, since many causes were unique in one or two experiments.
Figure 4. Primary study end-points. (A) survival curve, (B) clinical grading of hepatic encephalopathy, (C) blood ammonia levels (μmol/l). n=4 for LIS Control, Empty BAL and cBAL111 250 groups, n=1 for MPH 450 group. Average values (± SE) are reported.
Primary study end-points

Survival. Average survival times (Fig. 4A) were 9.0 ± 1.3 h for LIS Control group (n=4), 11.9 ± 1.8 h for Empty BAL group (n=4), 11.8 ± 0.9 h for cBAL111 250 group (n=4) and 19.1 h for MPH 450 group (n=1). Survival time of the cBAL111 250 group was significantly longer than the LIS Control group (P =0.026). However, the Empty BAL and cBAL111 250 groups did not differ in survival time (P = 0.595).

Neurological status. Neurological deterioration caused by failure of the ischemic liver was staged according to the clinical grading of hepatic encephalopathy (HE) (Fig. 4B). Animals were connected to the ex vivo bioreactor support system within 15 minutes after anesthesia was stopped. Within 45 minutes after LIS induction, all animals had recovered and were able to move freely in their cage. Grading of HE was performed by two observers and a HE status was given after mutual agreement. Importantly, to prevent any bias, grading was performed before blood ammonia levels were known at the indicated time point. All animals developed HE. No statistical differences were observed between the LIS Control, Empty BAL and cBAL111 250 group. The MPH 450 group was significantly different from the LIS Control (P = 0.001), Empty BAL (P = 0.006) and cBAL111 250 (P = 0.006) groups over the whole time period. Between 4 and 9 hrs, the LIS Control group tended to have higher HE grading scores as compared to the Empty BAL group, whereas the cBAL111 250 group tended to have lower HE grading scores.

Blood ammonia levels. Mean blood ammonia level of healthy rats (day 0, before PCS) was 28.3 ± 8 μmol/l and increased to 207 ± 79.1 μmol/l three days after the PCS. In the same period, the body weight of healthy rats (day 0, before PCS) was 350 ± 16 gram and decreased to 321 ± 15 gram (8.3 % loss of body weight). No significant differences were observed for blood ammonia levels between the LIS Control, Empty BAL and the cBAL111 250 group (Fig. 4C) after LIS was induced. However, a transient decrease in ammonia levels between 1 and 4 hrs after LIS was observed in the cBAL111 250 group. The MPH 450 group differed significantly from the LIS Control group (P = 0.018), and reached near significance with the cBAL111 250 group (P = 0.053) over the whole time period. No difference was found with the Empty BAL group.

Secondary study end-points

AST levels. AST levels were measured to confirm progressive liver damage after the LIS procedure. In general, AST levels increased in time to levels higher than 15000 U/L for all experiments (Fig. 5A). However, the time-course of AST release in the circulation differed between the individual experiments. As a result of this, the groups did not differ in the AST levels. There is a trend, however, for higher AST levels in the LIS Control group as compared to the other three groups, which may indicate a dilution effect when connected to the ex vivo bioreactor support system. Interestingly, AST levels remained
constant or even decreased after approx. 6 h, 8 h, 10 h and 6 h in the LIS Control, Empty BAL, MPH 450 and cBAL111 250 group, respectively. In one experiment in the cBAL111 250 group, we observed a prolonged survival time and continuous low HE grading in combination with low AST levels (< 15000 U/l). This led to an empirical exclusion criterion that all LIS rats should have AST levels higher than 15000 U/l in order to fulfill the criteria of a successful LIS.
Lactate metabolism. One hour after LIS, a ± 2-fold increase in lactate levels was observed for all groups which decreased to physiological levels at 3 h (Fig. 5B). After this initial peak, levels remained constant several hours, but then gradually increased. No significant differences were observed between all four groups.

Total bilirubin. For the Empty BAL, MPH 450 and cBAL111 250 group, total bilirubin levels gradually increased during the first 9 h with an average rate of 13.6 ± 0.8 μmol/l per hour ($P > 0.05$) (Fig. 5C). Total bilirubin levels of the LIS Control group, however, increased at a 3.6-fold lower rate of 3.8 μmol/l per hour. Total bilirubin constituted mainly of unconjugated bilirubin for all groups. These results indicate that cBAL111 as well as MPH bioreactors were unable to conjugate bilirubin to a significant level as compared to the Empty BAL group. Moreover, the large difference between the LIS Control group and the other three groups ($P = 0.010 - 0.043$) suggests the difference to be attributed exclusively to hemolysis, caused by the rotary pumps and the shear stresses of tubings, connectors and the plasmapheresis capillaries, as well as possible temporary large pressure differences over the plasmapheresis membranes (acute fouling).

Hemoglobin. Hemoglobin (Hb) levels decreased in time in all groups with an average rate of 0.42 ± 0.07 gr/dl for the Empty BAL, MPH 450 and cBAL111 250 group and 0.14 gr/dl for the LIS Control group (3-fold lower) during the first 9 h (Fig. 5D). Although these results are in accordance with the increase in total bilirubin, i.e. supporting the hypothesis of hemolysis, no significant differences existed between the four groups.

Creatinine. We determined the creatinine concentration to monitor the kidney function of the LIS rats as one of the parameters for multi-organ failure (MOF). During the first 9 h, creatinine increased at a rate of 5.7 μmol/l per hour for the LIS Control group, whereas the concentration of creatinine remained relatively stable (0.25 ± 0.33 μmol/l per hour) for the Empty BAL, MPH 450 and cBAL111 250 group (Fig. 5E). This significant difference may be attributed to a dilution effect of the ex vivo bioreactor support systems. After 9 h, creatinine levels strongly increased for the Empty BAL and cBAL111 250 group, whereas this effect was less pronounced for the MPH 450 group.

Hemodynamics. Mean arterial pressure (MAP) increased gradually during the first 9 hours (Fig. 5F). No statistical differences in that period were observed between all groups. However, the increase in the LIS Control group (7.3 mmHg per hour) was 1.4-fold higher than the average increase of the other three groups (5.3 ± 0.1 mmHg per hour).

Arterial blood gas analysis pH. No significant differences were observed between the Empty BAL, MPH 450 and cBAL111 250 group (Fig. 6A). However, the LIS Control group tended to have a lower blood pH during the ex vivo experiment as compared to the other three groups. In general, the pH increased within the first 3-5 h and remained relatively stable afterwards. In some cases the pH increased suddenly just prior to death, but this was not typical for one of the experimental groups.
Base excess. The base-acid ratio of the blood at a fixed pCO$_2$ is being expressed in the base excess (BE). In all groups, a typical trend was observed (Fig. 6B). BE tended to drop after 1 hour, after which it increased in 3 h to a stable high and positive level. Several hours before death, the BE decreased gradually, being the best predictor for coming death. Average BE levels differed between the groups and were the lowest according to the following ranking: LIS Control ($P < 0.05$) > Empty BAL ~ cBAL111 250 > MPH 450 ($P < 0.05$).

$pO_2$. Arterial oxygen pressure was strongly influenced by the oxygenation capacity of the bioreactor (Fig. 6C). Average pO$_2$ between 1 and 9 h was 98.8 mmHg for the LIS Control group (i.e. no bioreactor) and 134.6 ± 0.6 for the Empty BAL, MPH 450 and cBAL111 250 group ($P < 0.001$). High initial pO2 levels at $t = 0$ were caused by the anesthesia.

$pCO_2$. Arterial pCO$_2$ decreased in time for all groups and no significant differences were observed (Fig. 6D).
In vitro bioreactor tests - pre and post ex vivo

We expressed the function of the cBAL111 250 and the MPH 450 bioreactors as molar or U per hour per billion cells loaded, since the bioreactors were loaded with different amounts of cells. As such, the results of the pre-ex vivo, in vitro cBAL111 250 and MPH 450 bioreactor tests are in accordance with previously obtained results. When comparing the function of the cBAL 250 and MPH 450 bioreactors at the level of molar or U per hour per bioreactor, larger differences occur, due to the differences in cell number. These differences, however, are relevant in this model as the function of the whole bioreactor is decisive in the ex vivo experiment, and should, for that reason, be given first consideration. For this reason, we indicated the function of the cBAL111 250 and MPH 450, expressed as U per hour per bioreactor, in roman letters above each bar in the graphs as well.

Hepatocyte-specific functions

Ammonia elimination of the cBAL111 250 and MPH 450 bioreactors decreased similarly with a pre-post difference of 42 and 37.1 μmol/hours/billion cells, respectively (Fig. 7A).

![Graphs showing ammonia elimination, urea production, lidocaine elimination, and galactose elimination](image)

Figure 7. Hepatocyte-specific functions of the MPH 450 bioreactor (lined bars; n=1) and cBAL111 250 bioreactors (black bars, n=4) tested 1 day before ex vivo LIS period (pre) and directly after this period (post). (A) ammonia elimination, (B) urea production, (C) lidocaine elimination, (D) galactose elimination expressed in μmol/hour/10^9 viable hepatocytes, whereas values indicated above bars are values expressed in μmol/hour/bioreactor. Brackets indicate P < 0.05. Average values (± SE) are reported.
As a consequence, the MPH 450 bioreactor still eliminated ammonia post-
ex vivo at a level of 41% of the pre-test, whereas the cBAL111 250 bioreactors lost their capacity of ammonia elimination and instead, produced ammonia \( (P < 0.05) \). Urea production of the MPH 450 bioreactor decreased by 20 % post-
ex vivo, whereas a significant 16-fold increase was observed for the cBAL111 250 bioreactors (Fig. 7B). Lidocaine elimination of the MPH bioreactor decreased by 16 % post-
ex vivo (Fig. 7C). For the cBAL111 250 bioreactors, lidocaine elimination increased 1.4-fold, but remained less than 1 % of the elimination capacity of MPH 450 bioreactor. Galactose elimination remained stable for the cBAL111 250 bioreactors post-
ex vivo, whereas a 1.5-fold increase was observed for the MPH 450 bioreactor (Fig. 7C).

Global metabolic function and activity

Glucose consumption and lactate production remained relatively stable for the cBAL111 250 bioreactors pre versus post-
ex vivo (Fig. 8A and B). Glucose consumption increased by 2-fold and lactate production instead of elimination was observed in the MPH bioreactor post-
ex vivo in comparison to pre-
ex vivo. Oxygen consumption decreased by 32 % and 31 % for the cBAL111 250 and MPH 450 bioreactors, respectively (Fig. 8C).

Cellular integrity

AST release of the cBAL111 250 and MPH 450 bioreactors increased by 1.4-fold post-
ex vivo in comparison with pre-
ex vivo (Fig. 9A). LDH release remained stable in the cBAL111 250 bioreactors, whereas an 8-fold increased was observed in the MPH 450 bioreactor (Fig. 9B).

**Figure 8.** General metabolic function and activity of the MPH 450 bioreactor (lined bars; \( n=1 \)) and cBAL111 250 bioreactors (black bars, \( n=4 \)) tested 1 day before ex vivo LIS period (pre) and directly after this period (post). (A) glucose consumption, (B) lactate production, (C) oxygen consumption expressed in \( \mu \text{mol/hour/10}^9 \) viable hepatocytes, whereas values indicated above bars are values expressed in \( \mu \text{mol/hour/bioreactor} \). Bracket indicates \( P < 0.05 \). Average values (± SE) are reported.
Evaluation of cBAL111 in an ex vivo ALF rat model

Discussion

Recently, we have developed a new immortalized human cell line, cBAL111, as a possible alternative cell source for cell based therapies. In this study, we evaluated the potential of cBAL111 for BAL application inside the AMC-BAL in an animal ALF model using liver ischemic (LIS) rats as described earlier. We discuss the outcomes of this study and evaluate the model point-by-point and in more detail in Appendix 1 of this thesis entitled ‘Thoughts and Recommendations’.

With the current model, no improvement in the survival time of ALF rats and no differences in blood ammonia levels nor in the clinical grading of HE were observed between the cBAL111 250 and Empty BAL groups. The ex vivo bioreactor support system appeared to exert an intrinsic beneficial effect on the survival of LIS rats. This beneficial effect may have been caused by dilution of blood of ALF rats by redistribution of the extra-corporeal volume of the ex vivo bioreactor support system to the rat. This hypothesis is supported by the AST and creatinine results, showing a clear distinction between the LIS Control group and the other three groups that were connected to the extracorporeal system, i.e. Empty BAL, cBAL111 250 and MPH 450. For the blood ammonia and lactate concentrations, however, no indication of a dilution effect was present between the four groups. Treatment with the MPH450 bioreactor, as the golden standard for this model, appeared to have an advantageous effect on survival time, blood ammonia levels as well as the clinical HE score of ALF rats. Since only 1 experiment with MPH succeeded, no conclusions can be drawn with respect to the validity of this result.

One of the causes for the lack of therapeutic effect of cBAL111 bioreactors may be the detrimental effect of ALF plasma on the cBAL111 cells. ALF plasma is considered as potentially damaging to the cell because of the presence of toxic metabolites as well as harmful cytokines. However, studies investigating the effect of ALF plasma on various

Figure 9. AST and LDH release of MPH 450 bioreactor (lined bars; n=1) and cBAL111 250 bioreactors (black bars, n=4) tested 1 day before ex vivo LIS period (pre) and directly after this period (post). (A) AST release, (B) LDH release expressed in U/hour/10^9 viable hepatocytes, whereas values indicated above bars are values expressed in U/hour/bioreactor. Average values (± SE) are reported.
The effect of ALF plasma on cBAL111 and MPH was investigated by testing bioreactors loaded with either cell source before and after the ex vivo experiments in an in vitro setting.

Ammonia and lidocaine elimination as well as urea production and oxygen consumption of the MPH bioreactor decreased on average by 31.7 ± 13.9 %, whereas AST and LDH release increased after exposure of ALF plasma. Galactose elimination, glucose consumption and lactate production, on the other hand, appeared to follow the time-dependent trends as normally observed in in vitro tests of bioreactor cultures without any exposure to ALF plasma. These results indicate that ALF plasma has a particularly negative effect on the liver-specific functions of MPH, but affects not all cell functions. The extent to which ALF plasma affects the functional capacity of a BAL during the treatment of the LIS rat is, however, difficult to determine. The cell sampling technique as previously developed for the AMC-BAL might be a solution to determine time-dependent cellular processes during exposure to ALF.

ALF plasma had an important negative effect on the ammonia elimination capacity of cBAL111 bioreactors as well. cBAL111 bioreactors were able to eliminate ammonia before exposure to ALF plasma, but lost this capacity during the ex vivo experiment, resulting in a net production of ammonia post-ex vivo. Since cBAL111 bioreactors eliminate ammonia principally by the activity of glutamine synthetase (GS) and not by the urea cycle, ALF plasma may inhibit the activity or production of GS in cBAL111. Alternatively, increased amino acid catabolism of cBAL111 could also result in a net production of ammonia post-ex vivo. Metabolic flux analysis of cBAL111 under different culture conditions may reveal the causative mechanism. Since we only tested the functional capacity of cBAL111 before and after the ex vivo experiments, no conclusions can be drawn upon when cBAL111 lost the capacity of ammonia elimination during the ex vivo experiments. This probably occurred not in the initial phase because in the period between 1 and 4 h after inducing LIS, a transient blood ammonia removal was observed for the cBAL111 250 group in contrast to the Empty BAL group. Urea production of cBAL111 bioreactors increased post-ex vivo, possibly indicating an adaptive response of cBAL111 to the increased blood ammonia levels by restoration of the urea cycle enzymes. Analysis of mRNA levels of the urea cycle proteins of cBAL111 exposed to ALF plasma may confirm our findings. Such analyses may also provide us with further indications on how to efficiently induce urea cycle enzymes and cytochrome P450 activity.

In addition to the reduction in ammonia eliminating capacity after exposure to ALF plasma, the absence of beneficial effects of cBAL111 250 BAL treatment may be explained by the low urea production capacity and cytochrome P450 detoxification of cBAL111. To increase the therapeutic effect of cBAL111 in ALF, genetic engineering may provide cBAL111 with more liver-specific functional capacity, e.g. by overexpressing urea cycle and cytochrome P450 genes. An additional function to increase the potential of cBAL111 for BAL application is related to the inflammatory responses observed in ALF. Cytokines,
as stress mediators in ALF, have a direct impact on the function of hepatocytes.\textsuperscript{25} Shinoda \textit{et al.}\textsuperscript{26,27} have adopted this idea and investigated whether blockade of the interleukin-1 (IL-1) receptors would have a therapeutic potential. They transduced MPH with an adenoviral vector overexpressing human interleukin-1 receptor antagonist (AdIL-1Ra) and obtained improved survival times in a D-galactosamine (GalN)-induced ALF rat model after transplantation and BAL treatment.\textsuperscript{26,27} These experiments demonstrated that combining inflammatory cytokine blockade with a functional BAL device may be an effective therapeutic option in the treatment of ALF.

Another possible reason for the lack of beneficial effects of the cBAL111 250 BAL treatment may have been an insufficient number of cells per bioreactor. This study was started with 250 cBAL111 cells in the bioreactor, since the ammonia eliminating capacity, as determined by previous \textit{in vitro} BAL experiments\textsuperscript{9}, was calculated to be sufficient to yield a significant reduction in ammonia accumulation in the ALF rats. However, it should be realized that the total ammonia elimination capacity of cBAL111 250 bioreactors was app. 25\% of that of MPH 450 bioreactors, \textit{i.e.} 45\% fewer cells with 51\% less ammonia elimination capacity. Furthermore, the number of available cBAL111 cells was strongly limited by the cell culture capacity, as discussed earlier.

Since cBAL111 shows anaerobic glycolytic metabolism in the bioreactor, production of lactate may influence the physiology of ALF rats during an \textit{ex vivo} experiment. In this study, we did not observe increased plasma lactate levels in rats connected to cBAL111 250 bioreactors. On the other hand, an increased number of cBAL111 cells, \textit{e.g.} 450 million cells as with MPH 450 bioreactors, may lead to elevated blood lactate levels of the animal.

The ALF model used in this study was adopted from the original study of Flendrig \textit{et al.} which was performed approximately 8 years ago.\textsuperscript{3} Although the experimental set-up of this study was to large extent similar to the study of Flendrig \textit{et al.}, several differences in the methodology as well as in outcome parameters existed. The most prominent differences were observed in survival time and blood ammonia levels. Survival times of the LIS Control group and Empty BAL group from the study of Flendrig \textit{et al.} were 4.6 and 6.8 h shorter, respectively, as compared to this study. Blood ammonia levels in Flendrig’s study were generally higher and increased more dramatically within the first hour than in this study. Average ammonia levels at 1 h in Flendrig’s study was approx. 900-1000 \(\mu\text{mol/L}\), whereas this was approx. 500-650 \(\mu\text{mol/L}\) in this study. From these results, we conclude that the model used in the present study was milder than the study of Flendrig \textit{et al.} Possible reasons that could account for these differences might be related to the used animal strains, pre-operative period, anesthesia, plasmapheresis system, bioreactor type and configuration as well as fluid management. These issues and other possible relevant matters are further discussed in more detail in Appendix 1 of this thesis, entitled ‘Thoughts and Recommendations’.

In summary, we observed no improvement in the survival time of ALF rats and no
differences were shown in blood ammonia levels as well as in the clinical grading of HE between cBAL111 250 and Empty BAL groups. However, since we were not able to perform multiple successful experiments with MPH bioreactors, we cannot draw strong conclusions from this model (Fig 10). Nevertheless, future research will probably not use this model -valid or not-, since the current long survival times of both control groups increase the possibility of fatal errors or complications and drastically restricts use of the current model. Consequently, future research should focus on two aspects (related to this study): 1. improving the hepatocyte-specific function of cBAL111 by (a) introducing urea cycle genes\textsuperscript{28} and/or cytochrome P450 genes\textsuperscript{29}; (b) broaden the function of cBAL111 to a cell line with anti-inflammatory characteristics, e.g. by introducing human interleukin-1 receptor antagonist\textsuperscript{26,27}; (c) improving culture conditions to enhance general cellular metabolism\textsuperscript{15} as well as liver-specific functions of cBAL111; and 2. improving the ALF ex vivo model (see Appendix 1 ‘Thoughts and Considerations’ of this thesis).

**Figure 10.** Flow diagram for future work concerning cBAL111 and ALF model in rats. C/, conclusion.

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Reference List


