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Chapter 3

Assessment of the AMC-bioartificial liver in the anhepatic pig

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ABSTRACT

The anhepatic pig model was used to evaluate a bioartificial liver developed in our institution (AMC-BAL), based on oxygenated plasma perfusion of porcine hepatocytes attached to a polyester matrix.

Pigs (n=15) underwent total hepatectomy with restoration of caval continuity using a polyethylene, three-way prosthesis. In group I, pigs received limited intensive care under continuation of general anesthesia (n=5). Group II (n=5) underwent in addition, extracorporeal plasma perfusion of an AMC-BAL without hepatocytes (device control group). In group III (n=5), plasma perfusion took place of a BAL loaded with autologous hepatocytes. Groups II and III were connected to the extracorporeal system 24 hours after hepatectomy, for a period of 24 hours. Main outcome parameters were: survival time, liver enzymes (AST, ALT), blood ammonia and total/direct bilirubin.

Survival (mean ± SD) of the anhepatic pigs was significantly increased in the BAL-treated group (group III: 65 ± 15 hours), as compared to the control groups (group I: 46 ± 6 hours and group II: 43 ± 14 hours). Mean blood ammonia levels during BAL-treatment were significantly lower in the BAL-treated group in comparison to both control groups (p=0.02). Total and direct bilirubin levels gradually increased after hepatectomy and reached maximum values of 1.98 mg/dl and 1.50 mg/dl, respectively, showing no differences between the three groups.

We conclude that treatment of anhepatic pigs with the AMC-BAL containing autologous hepatocytes significantly increases survival time which is associated with a significant decrease in blood ammonia. Anhepatic pigs demonstrate increasing direct bilirubin levels as a result of extrahepatic bilirubin conjugation.

INTRODUCTION

Fulminant hepatic failure is a dramatic clinical syndrome, which is associated with significant mortality despite advances in medical management and liver transplantation(1-3). There is no question about the urgent need for liver-assist devices to bridge patients until a liver graft has become available for transplantation, or to provide liver support allowing the diseased liver to recover through parenchymal regeneration(4). We devised a new bioartificial liver (AMC-BAL) based on a bioreactor loaded with porcine hepatocytes. The bioreactor consists of a spirally wound, non-woven polyester matrix in a cartridge, in which the hepatocytes are allowed to attach to the matrix as described previously(5). The hepatocyte system is oxygenated by hollow-fibers that are integrated in the windings of the polyester matrix. The unique feature of this bioartificial liver is that the plasma of the patient has direct contact with the hepatocytes, thereby reducing the diffusion distance and allowing mass exchange similar to the situation in the sinusoids of the intact liver parenchyma.

It has been shown that the hepatocytes in the AMC-BAL generate metabolic function in in vitro studies(5). It also has been shown that the AMC-BAL achieves prolonged survival in the total liver ischemia model in rats(6), as well as in pigs(7). In the present study, the AMC-BAL was tested in a total hepatectomy model in pigs. This is a pure model of acute liver failure because there is simply no liver to sustain life. An
important difference with the liver ischemia model is the absence of the release of toxic products resulting from hepatic necrosis(8). The main objectives were to evaluate the AMC-BAL in this pre-clinical study design by assessing metabolic function and treatment efficacy in terms of extension of survival of the anhepatic animals.

MATERIALS AND METHODS

The anhepatic model in the pig
15 adult white female pigs were used, weighing between 37 and 57 kg (mean 48 ± 4.9 kg, no differences between groups). All procedures were approved by the institutional guidelines of the Animal Ethical Committee of the University of Amsterdam.

After fasting overnight, induction of anesthesia was achieved with intramuscular administration of ketamine (10mg/kg; Nimatec®, Eurovet, Bladel, the Netherlands), azaperoon (2 mg/kg: Stresnil®, Janssen Pharmaceutica, Tilburg, The Netherlands) and atropine (0.02 mg/kg). After inhalation of a mixture of O₂:NO₂ (2:3) and isoflurane (0.4-1%, Abbott Laboratories Ltd., Queensborough, UK), pigs were endotracheally intubated and ventilated with a mixture of O₂ and air. Anesthesia was maintained by intravenous administration (0.5 ml/kg/hour, after total heptectomy 0.2 ml/kg/hour) of a mixture of sufentanilcitrate (20mg/l, Janssen-Cilag, Tilburg, the Netherlands) and ketamine (20g/l). Muscle relaxation was obtained by intravenous administration (2ml/h) of pancuronium bromide (2 mg/ml, Organon Teknika B.V., Boxtel, the Netherlands). Arterial and venous lines were inserted in the right axillary artery and internal jugular vein for blood sampling and for continuous monitoring of arterial pressure and central venous pressure, respectively.

A laparotomy was performed using an upper abdominal midline incision. The liver was freed of all peritoneal attachments. The common bile duct and hepatic arteries were ligated and transected. The portal vein and caval vein were dissected free, with careful ligation of the remaining lymphatic tissue in the hepatoduodenal ligament. The portal vein was cross-clamped and cannulated (marking the start of the anhepatic state) after which the liver was flushed with 1000 ml of cold (4°C) Ringers glucose solution (NPBI, Emmer Compascuum, the Netherlands). After subsequent temporary clamping of the suprahepatic and infrahepatic caval vein, a self-made, rigid polyethylene, transparent three-way prosthesis was placed in the retrohepatic caval vein through a small venotomy and fixed cranially and caudally of the liver. The vascular prosthesis was flushed with heparin (10 U/ml, Leo BV, Weesp, The Netherlands) before use. Portal blood flow was shunted to the systemic circulation by connecting the portal vein to the side-port of the prosthesis in an end-to-side fashion. The time between clamping of the portal vein until restoration of blood flow in the portal and caval veins was 11-16 minutes. The liver was removed after a posterior longitudinal incision of the retrohepatic caval vein.

Postoperatively, all animals were kept under full anesthesia until death. Fluids were administered intravenously (electrolyte solutions and Ringers lactate, NPBI, Emmer Compascuum, The Netherlands; eloHaes, Fresenius BV, 's-Hertogenbosch, The Netherlands). Phenylephrine (10 mg/ml; 2-25 ml/h) was administered intravenously when necessary to maintain mean arterial blood pressure above 55 mmHg. Animals were kept on volume controlled, positive pressure ventilation during the experiment. No blood transfusions were given. Inspiratory peak pressure, capnography, direct arterial blood
pressure, and central venous blood pressure were monitored continuously and corrected to physiological values. Body temperature was maintained at 38° C using a heated mattress. 20 % glucose was infused when plasma glucose levels fell below 8 mmol/l. Every 12 hours, the animals received 1 gram of ceftriaxon iv (Roche, Basel, Switzerland). Post mortem examinations were performed in all animals.

Porcine hepatocyte isolation and preparation of the bioreactor.
Hepatocytes were isolated according to a modified method as described by Seglen(9), by perfusing the portal vein of the excised livers with 3 liters of an oxygenated Ca²⁺-free solution (37 Celsius) and a digestion buffer that contained 0,00625% (w/v) Liberase RH (Roche, Almere, The Netherlands), instead of collagenase (fig 1). The capsule of the liver was opened and the digested parenchyma was collected on ice after filtration through surgical gauze. The cell suspension was diluted with ice-cold Hanks’ buffer solution and washed 3 times through centrifugation at 4°C and 50g for 3 min followed by resuspension in Hanks’ buffer solution. Cell counts were determined after the third centrifugation. Finally, a last centrifugation was performed, after which the cells were suspended in culture medium. The culture medium consisted of Williams’ E medium, supplemented with heat inactivated fetal calf serum (10%(v/v), glutamin (2mM), insulin (1mIU/ml, Actrapid®, Novo Nordisk A/S, Bagsvaerd, Denmark), dexamethason (50 g/ml, Centrafarm (Etten-Leur, The Netherlands), penicillin (100U/ml), streptomycin (100 U/ml) and fungizone (0,25 g/ml, Diflucan®, Pfizer Inc. New York, USA). Williams’ E medium, fetal bovine serum, glutamine, and a mixture of penicillin, streptomycin and fungizone were obtained from BioWhittaker (BioWhittaker Europe, Verviers, Belgium). Isolated cells were transferred to the bioreactor and allowed to attach for 4-6 hours, while rotating (under oxygenated conditions) in a cabinet at 37 °C.

Total cell load of the bioreactor consisted of an average of 10.7 billion viable hepatocytes (range 7.9 -15.5 billion hepatocytes). Determination of viability was based on the trypan blue exclusion test. The bioreactor was subsequently connected to a closed perfusion system and perfused with 1750 ml recirculating culture medium over night.

BAL-system
The BAL-system and extracorporeal configuration used have been described previously(5;7). Briefly, the system (fig 2) consisted of two pump-driven, parallel circuits. The first circuit, the blood circuit, incorporated a centrifugation plasma-separator (Fresenius AS-104, Fresenius AG, Bad Homburg, Germany) after which the plasma was pumped through a second circuit including the bioreactor. Animals were connected to the plasma-separator via a double lumen catheter in the left jugular vein. Blood flow through the plasma-separator was 50 ml/min. Plasma was pumped through the bioreactor at 150 ml/min. Sodium citrate (11 g/l, citrate solution versus plasma 1:25) was added to the first circuit as an anticoagulant.

Experimental groups
Three experimental groups were studied. Group I served as absolute controls, and received limited intensive care after total hepatectomy (Control, n=5). Group II underwent in addition, plasma separation and plasma perfusion of an AMC-BAL without hepatocytes (Device Control, n=5). In group III, plasma perfusion took place of a BAL loaded with autologous hepatocytes (BAL-treatment, n=5). In groups II and III, extracorporeal
perfusion started 24 hours after hepatectomy, and was continued for a period of 24 hours. The animals of the different groups were mixed excluding any effects of a learning curve during the experiments.

Figure 1.
Schematic representation of total hepatectomy and use of the caval prosthesis, hepatocyte isolation, preparation of the bioreactor, and connection of the AMC-bioartificial liver via a plasma-separator unit to the systemic circulation of the animal.
Figure 2.
Schematic representation of the AMC-bioartificial liver system, consisting of a blood circuit (plasma obtained from the recipient by a centrifugal plasma-separator), and a plasma circuit in which the plasma perfuses the bioreactor. After recirculation through the bioreactor, the plasma is reunited with the blood cells from the plasma-separator and returned to the animal.

**Study parameters.**
Arterial blood samples were obtained preoperatively, at the moment of total hepatectomy (defined as t=0 h) and at 4 hourly intervals post-hepatectomy until death, for determination of AST, ALT, plasma lactate, blood urea nitrogen (BUN), direct and indirect bilirubin, electrolytes, hemoglobin, platelet count and prothrombin time. Ammonia was measured by means of a spectrophotometric method (ACA SX, Dupont).

**Statistics**
Results are presented as mean ± standard deviation. Data were analyzed using GraphPad Prism software (San Diego, CA). Analysis of variance was used to compare the three groups, and Bonferroni's multiple comparison analysis when overall effects were significantly different (p<0.05). Survival time was analyzed using the Kaplan Meier method, and significance was tested with the Log Rank Test (SAS 6.12, SAS Institute Inc., Cary, North Carolina, USA).
RESULTS

Surgical procedure
Total operation time was 145 ± 15 minutes. Blood loss during surgery was 275 ± 260 ml. This mainly represented the amount of blood, diluted with Ringer's glucose solution, contained in the liver before excision. Operation time and blood loss were equal for the three study-groups. Following total hepatectomy, all 15 pigs showed rapid stabilization of vital parameters and continued to have stable hemodynamical and ventilatory parameters for at least 24 hours.

Survival
Survival of the anhepatic pigs was significantly increased in the BAL-treated group (group III: 65.4 ± 15.4 hours) as compared to the control groups (group I: 45.8 ± 6.4 hours, p=0.022; group II: 42.6 ± 13.8 hours, p=0.036) (fig.3). Survival between both control groups was not significantly different (p=0.61). Longest survival recorded was 79 hours of an animal in the BAL-treated group. All animals died due to irreversible shock or cardiac arrhythmias.

At autopsy, 100-500 ml of serosanguinolent fluid was found in the abdominal cavity of all animals. The vascular prostheses were all patent, showing no signs of thrombosis or blood loss from the venous connections. No signs of portal congestion were noted.

![Figure 3](image)

**Figure 3.** Survival times of pigs after total hepatectomy (at t = 0 h) in the control group (dashed-dotted line), the device control group (dotted line) and the BAL-treated group (solid line). Survival of the anhepatic pigs was significantly increased in the BAL-treated group, as compared to groups I and II (p=0.022 and p=0.036). The black bar represents the time during which groups II and III were connected to the extracorporeal system.
Figure 4.
Arterial blood ammonia levels after total hepatectomy (at t = 0 h) in groups I (control), II (device control) and III (BAL-treated group). Ammonia levels were significantly lower in the BAL-treated group compared to both control groups. * Group I versus III, P < 0.01; † group II versus III, P < 0.05; § group I versus III, p<0.05. The black bar represents the 24-hours period in which animals of groups II and III were connected to the extracorporeal system. Results are shown as means ± SD, calculated according to the number of animals that were alive at the indicated time points.

Figure 5.
AST and ALT levels in pigs after total hepatectomy (at t = 0 h). The black bar represents the 24-hours period in which animals of groups II and III were connected to the extracorporeal system. Results are shown as means ± SD, calculated according to the number of animals that were alive at the indicated time points.
Figure 6.
Direct (A) and total bilirubin (B) levels in pigs after total hepatectomy (at t = 0 h) in the three study groups. The black bar represents the 24-hours period in which animals of groups II and III were connected to the extracorporeal system. Results are shown as means ± SD, calculated according to the number of animals that were alive at the indicated time points.
Biochemical parameters
Blood samples drawn at 24 hours after total hepatectomy (which was just before groups II and III were connected to the plasma separator), showed no significantly different values of the biochemical parameters between the three groups (i.e. levels of hemoglobin, blood ammonia levels (p=0.065 between groups I vs. III), blood urea nitrogen, albumin, AST/ALT, electrolytes, plasma lactate, prothrombin time and platelet count).

In the first 24 hours after hepatectomy, a trend of lower blood ammonia levels in group III was observed, with significant differences at 3 and 16 hours posthepatectomy. At 44 and 48 hours post-hepatectomy, significantly lower levels of ammonia were seen in group III as compared to both control groups (Fig.4).

AST levels in all groups showed a moderate rise after hepatectomy, resulting from the surgical procedure of total hepatectomy (Fig 5). In group III, one animal showed high AST levels after connection to the AMC-BAL (maximum 1352 U/l at 28 hours), probably due to leakage of AST out of suboptimal hepatocytes in the bioreactor. Remarkably, the survival time (41 hours) of the animal connected to this particular bioreactor fell in the range of the control-group. Plasma ALT values were not different from base line activity. Plasma lactate levels and BUN did not differ between groups and rose to a maximum of 5.9 ± 3.9 mmol/l and 10.7 ± 5.5 mmol/l, respectively, at death. In both control groups and BAL-treatment group, the prothrombin time exceeded 40 seconds at 20 hours after hepatectomy and did not change during BAL-treatment.

Both total and direct bilirubin levels gradually increased after total hepatectomy and reached maximum values of 1.98 mg/dl and 1.50 mg/dl, respectively, showing no differences between the three groups (Fig. 6).

DISCUSSION

This study describes application of the AMC-BAL in pigs with acute liver failure based on total removal of the liver. Anhepatic animals treated with the AMC-BAL device showed reduced blood ammonia levels and significantly longer survival times.

Total hepatectomy as a model of acute hepatic failure has been studied since 1921, when Mann and Magath used a glass tubing between portal and caval veins to reconstitute flow after total hepatectomy in the dog(10). Since then, several methods of total hepatectomy have been described, in which the liver is either dissected off the vena cava in association with a portocaval shunt(11), or in which the liver is removed including the portal and caval veins which are replaced by a prosthetic graft(12-18). After surgery, the animals in these studies were allowed to wake up and breath spontaneously. Mean survival times in these studies ranged from 12 to 30 hours(12;14). Postoperative care of the anhepatic animals in our present study included mechanical ventilation, which probably is an important factor attributing to the longer survival times of the control group in the present study (mean 46 hours, maximum 51 hours) when compared to the above-mentioned studies using the total hepatectomy model in the pig.

There is a long history in medical literature relating to the development of various temporary liver-support therapies. In 1958 hemodialysis was introduced as a first attempt to remove toxins thought to cause hepatic encephalopathy(19). Other groups introduced resin(20) or activated charcoal perfusion(21), which were also used as detoxification therapy in liver failure. Although these artificial therapies showed
significant improvements in biochemical profile and neurological status in patients with severe liver failure(22), no significant improvement of survival could be demonstrated. Eiseman reported the first clinical application of extracorporeal porcine whole liver perfusion in 1965(23), and this concept was followed by others(24). Although some of these studies showed neurologic improvement of patients, no controlled trials have been performed showing prolonged survival(24). A survival of up to 57% in noncontrolled studies was described using baboon and cadaveric livers(25).

Realizing that full substitution of hepatocyte function may be necessary for treatment of liver failure, many research groups have focused on the use of isolated hepatocytes as the basis for liver support. Extracorporeal bioreactors were designed capable of containing functional hepatocytes that would support liver functions by exchanging plasma components with the patient. A small number of such hybrid or bioartificial livers are currently being studied in clinical phase II/III trials. These are all based on semipermeable hollow-fiber membranes, in which the patient’s plasma flows through the lumen of the hollow-fibers, and hepatocytes are cultured in the extracapillary space. The Berlin Extracorporeal Liver Support System (BELS), introduced by Gerlach et al.(26)) consists of three independently woven capillary membrane systems in between which the porcine hepatocytes reorganize as aggregates. Each membrane system serves a different function: plasma inflow, plasma outflow and cell oxygenation. Treatment of anhepatic pigs with the BELS containing 10 billion pig hepatocytes showed an improvement in blood ammonia, phenylalanine and lactate levels, but there was no survival benefit compared to the control group(27). The Extracorporeal Liver Assist Device (ELAD), introduced by Sussman et al. utilizes four hollow fiber cartridges each containing approximately 100 grams of hepatocytes from the C3A human hepatoblastoma cell line. Animal studies in six anhepatic dogs showed improved metabolism of anesthetics in the animals, but no prolongation of survival(28). The HepatAssist is a porcine hepatocyte-based bioartificial liver, which was developed by Demetriou and coworkers(29). In this system containing five billion cryopreserved, microcarrier attached hepatocytes, a charcoal column is added to the extracorporeal perfusion circuit. Dogs with complete liver ischemia treated with the HepatAssist showed significant neurological and biochemical improvements, but life could not be prolonged(30). In a phase I clinical trial, 31 patients in three groups were treated with the HepatAssist(31). In the first group of fulminant hepatic failure patients, 16 were successfully bridged to liver transplantation and one spontaneously recovered without a transplant; in the second group, all three patients with primary nonfunction of a transplanted liver were successfully bridged to retransplantation; in the third group of 10 patients with acute exacerbation of chronic liver diseases only two were supported to recovery and successful transplants at later dates, the other 8 patients were not eligible for transplantation. A large multicenter phase II/III clinical trial is currently under way.

In a previous study using pigs with induced total liver ischemia, the AMC-BAL likewise showed significantly prolonged survival of animals after 24h treatment with the AMC-BAL when compared to controls(7). Moreover, in this liver ischemia model, blood ammonia levels and total bilirubin levels decreased significantly during treatment, indicating metabolic activity of the porcine hepatocytes in the bioreactor. The total liver ischemia model is a surgical model of acute hepatic failure. It resembles clinical fulminant hepatic failure to some extent, in that the failing liver remains in situ and is allowed to release products of necrosis in the systemic circulation, which allegedly are responsible for the toxic syndrome of acute liver failure. This could explain the longer survival in the
present (anhepatic) control group compared to the control group with total liver ischemia in our previous study (46 ± 6 hours versus 33 ± 3 hours, respectively)(7).

The anhepatic model is a surgical, pure model of hepatic failure. Advantages of this model are its clarity and its potential to assess in vivo biochemical capacity of a bioartificial liver device in the absence of toxic products leaking out of, or produced by the native liver(18). Obviously, the release of products of necrosis and signal transmitters, seen as an important factor in the pathophysiology of acute liver failure, is absent in the anhepatic model(8;32;33). Our data show that the employed technique of total hepatectomy in pigs results in a well-reproducible model of acute liver failure. The method yields an intact liver for autologous cell isolation purposes and involves a relatively minor surgical trauma as demonstrated by the rapid stabilization of the animal after surgery. Postoperative care including limited intensive care such as mechanical ventilation offers a therapeutic window suitable for assessment of treatment with a bioartificial liver.

The survival benefit of anhepatic pigs treated with the AMC-BAL indicates significant functional capacity of the bioartificial liver. The exact processes that are involved during liver support remain unclear, in as much as our understanding of the pathophysiology of liver failure is incomplete(8;32;33). Blood ammonia levels in BAL-treated animals were significantly decreased at the end of the treatment period. An interesting observation is the persistence of low levels of blood ammonia in the post-treatment period. This was also found in the above-mentioned, previous study in which the AMC-BAL was used in pigs with liver-ischemia(7). Possibly, the persistent low levels of ammonia owe to a prolonged effect of the BAL or an improved metabolic state of the animals.

The gradual and continuous rise of plasma direct-bilirubin in the anhepatic pigs indicates an alternative pathway of extrahepatic conjugation of bilirubin. UDP-glucuronosyltransferases, capable of bilirubin conjugation, have been shown to be present in liver, kidney and intestinal mucosa of humans(34;35), rats(36;37) and pigs(38). The large capacity of extrahepatic bilirubin metabolism as seen in the present study has not been described before.

In conclusion, treatment of anhepatic pigs with the AMC-BAL liver significantly increased survival time in association with a significant decrease in blood ammonia. These results are comparable with previous studies using the AMC-BAL in pigs with total liver ischemia(7). To our knowledge, there are no data in literature of other liver support systems that have shown significant prolongation of life in large animals with hepatic failure. The rise of direct bilirubin indicates the ability of extrahepatic conjugation of bilirubin in the anhepatic animals. These results encouraged us to set up a phase I clinical trial with the AMC-BAL in patients with fulminant liver failure.
References


