Experimental treatment modalities for liver failure
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Chapter 4

Blood Coagulation in Anhepatic Pigs: Effects of Treatment with the AMC- Bioartificial Liver

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ABSTRACT

The function of a newly devised bioartificial liver (AMC-BAL) based on viable, freshly isolated porcine hepatocytes has been evaluated in anhepatic pigs. The aim of this study was to assess the contribution of BAL treatment on blood coagulation parameters.

Pigs were anaesthetized and a total heptectomy was performed (n=15). The infrahepatic caval vein and the portal vein were connected to the subdiaphragmatic caval vein using a 3-way prosthesis. Animals received standard intensive care (Control, n=5), treatment with an empty BAL (Device Control, n=5) or with a cell-loaded BAL (BAL-treatment, n=5) for a period of 24 hours starting 24 hours after heptectomy. Coagulation parameters studied concerned prothrombin time (PT), platelet count, the procoagulant system (factors II, V, VII, VIII and fibrinogen), anticoagulant system (AT III), fibrinolytic system (t-PA, PAI-1) as well as markers of coagulation factor activation (TAT complexes, prothrombin fragment F1+2).

Factors II, V, VII, AT III and fibrinogen rapidly decreased after total heptectomy in pigs in accordance with the anhepatic state of the animals. Factor VIII levels were not influenced by the heptectomy. A mild drop in platelet count was seen in all groups. Treatment of anhepatic pigs with the cell-loaded BAL did not restore PT or clotting factor levels. TAT and F1+2 complexes however, were significantly increased in this group. Levels of t-PA and PAI-1 were not influenced by cell-loaded BAL treatment.

Treatment of anhepatic pigs with the AMC-BAL based on freshly isolated porcine hepatocytes does not result in an improved coagulation state due to extensive consumption of clotting factors. However, increased levels of TAT complexes and prothrombin fragments F1+2 during treatment of anhepatic pigs indicate synthesis and direct activation of coagulation factors, leading to thrombin generation. This demonstrates that this bioartificial liver is capable of synthesizing coagulation factors.

INTRODUCTION

The liver plays a central role in the regulation of blood coagulation. Hepatocytes synthesize almost all coagulation factors and coagulation inhibitors as well as components of the fibrinolytic system. In addition, hepatic clearance of activated clotting proteases and protease-protease inhibitor complexes importantly modulates coagulation activation. Consequently, liver failure is always accompanied by a hemostatic defect. Blood coagulation can be severely compromised especially in patients with fulminant hepatic failure (FHF), potentially leading to major hemorrhage and possible systemic activation of coagulation (DIC).

A bioartificial liver (BAL) is a support system based on viable hepatocytes incorporated in an extracorporeal plasma circuit, intended to bridge patients with FHF to transplantation or liver regeneration. A small number of such hybrid or bioartificial livers is currently being studied in clinical phase II/III trials (1-3). As liver function is (at least in part) replaced by a BAL, one might assume that successful treatment with a BAL influences the hemostatic system in such a way that blood coagulation defects could be more or less corrected.
A bioartificial liver (AMC-BAL) has been devised in our institution and is based on a hollow fiber bioreactor loaded with freshly isolated porcine hepatocytes. The bioreactor consists of a spirally wound, non-woven polyester matrix to which the hepatocytes attach. In between the layers of the matrix hollow fibers for oxygen transport are situated for direct oxygenation of the hepatocytes inside the bioreactor. In the AMC-BAL there is a direct contact between plasma of the patient and the hepatocytes, unlike most other, membrane-based bioartificial liver systems. It has been shown in vitro that the hepatocytes in the AMC-BAL provide metabolic function(4). Furthermore, it has been demonstrated that the AMC-BAL prolongs survival in acute liver failure models in rats(5) as well as in pigs(6;7). In several animal studies, an improvement of the coagulation status during treatment with a bioartificial liver has been shown(8-11). In these studies, ischemic models of fulminant hepatic failure were used in rats and pigs, with or without partial hepatectomy. It remains unclear whether these better coagulation parameters were due to production of coagulation factors in the bioreactor, or due to faster functional recovery of the remaining (ischemic) liver.

Although the inclusion of a biological component in a liver support system derives from the assumption that a synthetic function is required next to plasma detoxification, there is little proof that hepatocytes in a BAL provide vital synthetic products. The aim of this study was to assess the contribution of the AMC-BAL to blood coagulation, during treatment of anhepatic pigs.

MATERIALS AND METHODS

The anhepatic model in the pig
Pigs (37-57 kg) were anaesthetized and a total hepatectomy was performed (n=15). The infrahepatic caval vein and the portal vein were connected to the subdiaphragmatic caval vein using a 3-way vascular prosthesis. Postoperatively, all animals were kept under full anesthesia and mechanical ventilation until death. No blood transfusions, platelets, fresh frozen plasma or other substances containing coagulation factors were given during the experiment. Fetal bovine serum (FBS) is present in the culture medium used to load the hepatocytes in the bioreactor (10% v/v in 450 ml), but this is washed out of the bioreactor before connecting the system to the anhepatic pig using 4500 ml of Shiwa (B Braun, Glandorf, Germany) supplemented with 20 mU/ml Actrapid (Novo Nordisk, Bagsvaerd, Denmark), 1 M dexamethason-dinatriumphosphate (Centrafarm services B.V., Etten-Leur, The Netherlands), 5% sodium citrate (ACD-Formula A; Emmer Compascum, The Netherlands) and 20 g/l human albumin (CLB, Amsterdam, The Netherlands). Body temperature was maintained at 38° C using a heating mattress.

The BAL-system and experimental groups
Autologous hepatocytes were isolated from the excised liver using a modified isolation procedure(12). Calcium free medium (2000 ml) was flushed through the liver via the portal vein followed by recirculating perfusion with Liberase-RH (Roche, Almere, The Netherlands) solution. After washing the harvested hepatocytes using three centrifuginal (50g) washing steps, the cells were loaded into the bioreactor. The BAL-system and extracorporeal configuration used in this study have been described previously(4;6;7). Briefly, the system consists of two pump-driven, parallel circuits. The first circuit, the
blood circuit, incorporates a centrifugation plasma-separator (Fresenius AS-104, Fresenius AG, Bad Homburg, Germany) after which the plasma is pumped through a second circuit including the bioreactor. 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. Sodium citrate (11 g/l, citrate solution versus plasma 1:25) was added to the first circuit as anticoagulant.

After 24 hours of anhepatic state, the animals were divided into three groups. The first group served as absolute control, and received limited intensive care, including mechanical ventilation and vasopressors, after total hepatectomy (Control, n=5). The second group underwent plasma separation and plasma perfusion through a bioreactor without hepatocytes for the next 24 hours (Device Control, n=5). In the third group, plasma perfusion took place through a bioreactor loaded with autologous hepatocytes for the next 24 hours (Cell-loaded BAL-treatment, n=5).

Study parameters.
Blood and citrated plasma samples were collected preoperatively, and at 4 hourly intervals until 48 hours after hepatectomy. Platelets were counted by flow cytometry. Antithrombin III (AT III) was measured using an amidolytic assay. Prothrombin time (PT), factor II, V, VII, VIII and fibrinogen were determined using one stage clotting assays with human factor II, V, VII and VIII deficient plasma and a human fibrinogen standard and Thromborel-S thromboplastin (Dade Behring, Leusden, The Netherlands). Thrombin-antithrombin III (TAT) complexes, prothrombin fragment F1+2, tissue-type plasminogen activator (t-PA) and plasminogen activator inhibitor type I (PAI-1) were determined using ELISA’s (Behring, Marburg, Germany). F1+2, TAT, t-PA and PAI-1 were measured in samples collected at 0, 8, 20, 24, 28, 32 and 48 hours after hepatectomy.

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).

RESULTS

Anhepatic model
No complications were encountered during the surgical procedure of performing a total hepatectomy. Total operation time was 145 ± 15 minutes. Blood loss during surgery was 275 ± 260 ml. These parameters were equally distributed between 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.

BAL-treatment
The outcome of the anhepatic animals treated with the BAL has been described elsewhere(7). Briefly, survival (mean ± SD) of the anhepatic pigs was significantly increased in the BAL-treated group (65 ± 15 hours), as compared to the control groups.
(Control: 46 ± 6 hours and Device Control: 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). All animals showed diffuse, minor bleeding from operation wounds and introduction sites of intravascular catheters after approximately 24 hours postoperatively. All animals received moderate intensive care treatment until death. At autopsy, 100-500 ml of serosanguinolent fluid was found in the abdominal cavity of the animals. No signs of major bleeding were identified.

**Figure 1.**
Prothrombin time and platelet count in anhepatic pigs after total hepatectomy (at t = 0 h) in the three study groups. Upper limit of measurement is 40 seconds. Data are expressed as means ± SD. The black bar indicates the 24-hours period in which animals of the Device Control and BAL-treated groups were connected to the extracorporeal system.
Factors II, V, VII and AT III in anhepatic pigs after total hepatectomy (at t = 0 h) in the three study groups. Data are expressed as means ± SD. The black bar indicates the 24-hours period in which animals of the Device Control and BAL-treated groups were connected to the extracorporeal system.
Coagulation parameters
Mean prothrombin time gradually increased after total hepatectomy, reaching the upper limit of measurement (40 seconds) after 32 hours in all three groups. Mild thrombocytopenia occurred without significant differences between groups (Fig. 1).

Coagulation factors II, V and VII, fibrinogen and antithrombin III showed a rapid reduction after total hepatectomy, without any effect of treatment with the BAL (Fig. 2; fibrinogen data not shown). The decrease of factor VII preceded that of factor II and V, which is consistent with the short (6 hours) biological half-life of factor VII. Levels of factor VIII, the only blood coagulation factor not exclusively produced by the liver, showed considerable variation between the animals, but overall, was not affected by the anhepatic state and/or the AMC-BAL treatment (Fig. 3).

Thrombin generation, as reflected by prothrombin activation fragment F1+2 and thrombin-antithrombin (TAT) complexes, increased from 1.4 to 135 nmol/l and from 11 to 344 g/l, respectively, following hepatectomy. After reaching peak values after 20 hours, these markers rapidly declined but not in the group treated with the AMC-BAL. In this group, F1+2 and TAT levels remained elevated up to the end of the experiment (Fig. 4), indicating de novo synthesis and immediate activation of prothrombin.

Tissue-type plasminogen activator (t-PA) reached levels one hundred-fold higher than baseline values and was not affected by BAL or control treatments. Plasma levels of the fibrinolytic inhibitor, PAI-1, showed a concurrent initial increase from 11 to 64 U/ml at 24 hours after hepatectomy and hereafter rapidly decreased in all groups (Fig. 5).

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**Figure 3.**
Factor VIII in anhepatic pigs after total hepatectomy (at \( t = 0 \) h) in the three study groups. Data are expressed as means ± SD. The black bar indicates the 24-hours period in which animals of the Device Control and BAL-treated groups were connected to the extracorporeal system.
Figure 4.
TAT complexes and prothrombin fragment F1+2 in anhepatic pigs after total hepatectomy (at t = 0 h) in the three study groups. Data are expressed as means ± SD. * both control groups versus BAL-treated P < 0.05; † both control groups versus BAL-treated P < 0.01; § both control groups versus BAL-treated P < 0.001. The black bar indicates the 24-hours period in which animals of groups II and III were connected to the extracorporeal system.

DISCUSSION

A bioartificial liver, serving as a liver-assist device, has a large potential 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(13). In contrast to many other liver support treatments like hemodialysis, hemoperfusion or albumin
dialysis, a bioartificial liver is assumed to replace liver specific synthetic function next to detoxification of the plasma. Viable hepatocytes present in a BAL are supposed to replace or support the patient’s multiple and complex own liver functions during liver failure, encompassing both detoxification and synthesis. Previous studies using the AMC-BAL in rats and pigs with severe liver failure showed reduced blood ammonia levels, reduced total bilirubin levels and significantly prolonged survival (5-7). In this study, we tested the hypothesis that treatment with the biologically active AMC-BAL supports blood coagulation in a model of acute liver failure.

A total heptectomy was performed to induce acute hepatic failure in pigs. Synthesis of procoagulants and coagulation inhibitors are efficiently eliminated in this model, as well as the capacity of the liver to clear activated coagulation proteins. A situation of total heptectomy allows assessment of the pathophysiology of hepatic failure and the effects of treatment with a BAL, without interference of the release of products of necrosis of hepatocytes into the systemic circulation. We observed a rapid decline of coagulation factors produced by the liver. During AMC-BAL treatment, sustained high levels of TAT complexes and prothrombin fragment F1+2 were seen most likely derived from direct activation and complexation of newly synthesized coagulation factors in anhepatic pigs. These data indicate that BAL treatment indeed results in synthesis of coagulation proteins. It is less probable that this is due to activation of the coagulation by the BAL device itself, as the control group and the device control groups show the same results. However, these newly synthesized factors merely serve as a substrate for the ongoing activation of coagulation set forth partly by the surgical trauma of heptectomy and as a consequence of the induced liver failure. They are not sufficient to restore clotting factor activity to a measurable amount. How this translates to the clinical setting of fulminant liver failure is a matter of speculation. The synthesis of coagulation proteins possibly contributes to improvement of hemostatic function. Alternatively, newly synthesized factors may serve to fuel ongoing systemic activation of coagulation, which can potentially be detrimental. We did however not find any evidence of systemic, macrovascular thrombus deposition in BAL-treated animals. While both prohemostatic and antihemostatic mechanisms were affected 24 hours after total heptectomy, overt diffuse bleeding from operation wounds and skin lesions was seen, along with an increase of PT. A mild thrombocytopenia was apparent in all groups. Worth mentioning is the absence of splenomegaly or other signs of portal hypertension at autopsy. Although an acute, acquired platelet dysfunction cannot be excluded, the decrease in platelet count is probably clinically insignificant. Coagulation factors II, V, VII and fibrinogen decreased to very low levels, due to the anhepatic state of the animals. Factor VIII levels were not affected, probably because this clotting protein is not produced exclusively by liver parenchymal cells (14). The increase and subsequent decrease of plasminogen activity inhibitor-1 is compatible with the activation of the coagulation system in the direct postoperative period, followed by a depletion of coagulation factors. The rise in t-PA levels, indicating fibrinolytic activity, has also been observed during the anhepatic phase in human liver transplantation (15). Fibrin degradation products (FDP) were not measured, since this assay is not only affected by thrombin generation but also by fibrinolytic activity. Since fibrinolysis is strongly activated in the anhepatic pig (due to very high levels of plasminogen activator and low levels of plasminogen activator inhibitor), FDP’s are likely to be very high in both situations. Similarly, soluble fibrin levels will increase both in case of increased clotting factor synthesis by the BAL and if the BAL itself will activate coagulation.
In conclusion, increased levels of TAT and F1+2 complexes in anhepatic pigs treated with the AMC-BAL demonstrate release (and most likely synthesis) of coagulation products by the bioreactor. No effect on other blood coagulation parameters was seen, presumably due to direct consumption of coagulation factors derived from the AMC-BAL during treatment of the anhepatic pigs.

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**Figure 5.**

$t$-PA and PAI-1 in anhepatic pigs after total hepatectomy ($t = 0$ h) in the three study groups. Data are expressed as means ± SD. The black bar indicates the 24-hours period in which animals of groups II and III were connected to the extracorporeal system.
References


