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CHAPTER 8

Effects of acute-liver-failure-plasma exposure on hepatic functionality of HepaRG-AMC-Bioartificial Liver

Submitted

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ABSTRACT

Background: The AMC-bioartificial liver loaded with the human hepatoma cell line HepaRG as biocomponent (HepaRG-AMC-BAL) has recently proven efficacious in rats with acute liver failure (ALF). However, its efficacy may be affected by cytotoxic components of ALF plasma during treatment.

Aims: The aim of this study was to investigate the effects of ALF-plasma on the HepaRG-AMC-BAL.

Methods: HepaRG-AMC-BALs were connected to the blood circulation of rats with total liver ischemia, either during the first 5 hours after induction of ischemia (mild ALF group), or during the following 10 hours (severe ALF group). After disconnection, the BALs were assessed for cell leakage, gene transcript levels, ammonia elimination, urea production, cytochrome P450 3A4 activity, apolipoprotein A 1 production, and amino acid metabolism.

Results: Cell leakage increased 2.5-fold in the severe ALF group, but remained limited in all groups. Hepatic gene transcript levels decreased (max 40-fold) or remained stable. In contrast, hepatic functions increased slightly or remained stable. Particularly, urea production increased 1.5-fold, with a concurrent increase in arginase 2 transcription and arginine consumption, whereas there was a trend towards a reduction in the conversion of ammonia into urea.

Conclusions: These results indicate that the HepaRG-AMC-BAL retains functionality after both mild and severe exposure to ALF plasma, but urea production may be increasingly derived from arginase 2 activity instead of urea cycle activity. Nonetheless, the increase in cell leakage and decrease in various hepatic transcript levels suggest that a decrease in hepatic functionality may follow upon extended exposure to ALF plasma.
**INTRODUCTION**

Bioartificial livers (BALs) have been developed to bridge patients with acute liver failure (ALF) and acute-on-chronic liver failure to liver transplantation or liver regeneration.1 ALF and acute-on-chronic liver failure differ in the pre-existence of liver disease, but are both characterized by massive hepatocellular necrosis leading to, most prominently, hepatic encephalopathy and multi-organ failure.2 BALs consist of a bioreactor loaded with liver cells that can be connected to the circulation of these patients to replace their failing liver. A major roadblock to clinical application of a BAL is the lack of a human liver cell line that displays high all-round hepatic functionality. The human hepatoma cell line HepaRG is a promising candidate in this respect, as it displays this high hepatic functionality in vitro, particularly after culturing in the AMC-BAL.3 Treatment with this HepaRG-AMC-BAL increased the survival of rats with ALF.3-7 To achieve optimal performance of the HepaRG-AMC-BAL in a clinical setting, it is essential that its functionality is maintained during exposure to the toxic plasma of patients suffering from ALF or acute-on-chronic liver failure. Particularly ALF plasma contains high levels of hepatotoxic compounds that might affect BAL functionality, including constituents of necrotic cells, cytokines produced by a deranged immune response, lipopolysaccharides, and compounds that are normally detoxified by the liver, such as ammonia, lactate, and bile acids.8-12 All these toxins can exert detrimental effects to the cells applied in the BAL, including loss of cell-cell contacts, a decrease in DNA and protein synthesis, a decrease in glutathione production, apoptosis and necrosis, and, most importantly, a decrease in hepatic functionality.13-16 Hepatic functions described to be decreased in this respect include ammonia elimination, urea production, and cytochrome p450 (CYP)-mediated detoxification.17, 18 Interestingly however, an upregulating effect of ALF plasma on hepatic functions has also been reported. Two studies demonstrated an increased urea production after exposure of freshly isolated hepatocytes to ALF plasma, and one also reported increased gluconeogenesis and albumin synthesis after ALF-plasma exposure.19, 20

Recently, we have investigated the effects of 8 to 16 hours exposure to ALF plasma (harvested from rats after death from ALF) on HepaRG cells cultured in monolayer. After ALF-plasma exposure, we observed an increase in cell damage and a downregulation of various hepatic genes (Hoekstra R et al, in press). However, we also demonstrated that the ammonia elimination rate was relatively unaffected in the HepaRG cultures; and while the conversion of ammonia into urea decreased, the total urea production increased upon ALF-plasma incubation. This discrepancy is most likely explained by the observed increase in arginase 2 expression, the gene coding for arginase 2, an enzyme that is – in contrast to cytosolic arginase 1 – not part of the urea cycle, as it is located in the mitochondria, where it also catalyzes the conversion of arginine into urea and ornithine.21

However, the effects of ALF plasma are not only dependent on the cell type, the composition of the ALF plasma and duration of ALF-plasma exposure, but also on the culture conditions of
the cells. As the culture conditions on monolayer are profoundly different from those inside the AMC-BAL (3D environment, continuous medium perfusion and oxygen supply), HepaRG cells might respond differently to ALF-plasma exposure when cultured in the AMC-BAL. Moreover, the effects of ALF-plasma exposure on several other hepatic functions of HepaRG cells, such as detoxification, synthetic functions, and lactate consumption, have not been assessed yet.

Therefore, the aim of this study was to assess the effects of ALF-plasma exposure on the performance of the HepaRG-AMC-BAL. To this end, we tested cell damage, transcript levels of various hepatic genes, and a number of liver-specific functions of HepaRG-AMC-BALs in a rat model of total liver ischemia. BALs were tested prior to connection to the rats (control group), after 5 hours of exposure to rats developing mild ALF (mild ALF group), and after 10 hours of exposure to rats developing from mild ALF to death (severe ALF group).

METHODS

**HepaRG-AMC-BAL Culture**

HepaRG cells were kindly provided by prof. C. Guguen-Guillouzo (INSERM, Rennes, France) and, for expansion, the cells were cultured in Hyperflasks (Corning, New York, U.S.) in HepaRG culture medium without dimethyl sulfoxide (DMSO). Laboratory scale versions of the AMC-BAL (9 mL volume) were loaded with ~750 million HepaRG cells and subsequently cultured in HepaRG culture medium supplemented with 1mM N-carbamoyl-L-glutamate (Sigma Aldrich, St. Louis, U.S.) and in absence of DMSO, as described previously.

**Treatment of ALF rats**

ALF rats were treated with HepaRG-AMC-BALs in an extracorporeal BAL system. All procedures were conducted in accordance with the institutional guidelines of the local Animal Ethical Committee and the animals received humane care. In short, a model of complete liver ischemia was used to induce ALF in Wistar rats. Rats were given a porto-caval shunt and three days later the hepatic artery was ligated to induce complete liver ischemia and thereby ALF. One hour after induction of ALF (t = 1 hour), the BAL treatment was started by perfusing the rat plasma through a HepaRG-AMC-BAL. The first HepaRG-AMC-BAL was disconnected from the extracorporeal BAL system at t = 6 hours, after 5 hours of treatment, and replaced by a second one. This second HepaRG-AMC-BAL was used to treat the rat until death occurred (between t = 15 hours and t = 16 hours). This setup was chosen to study the effects of both mild and severe ALF plasma exposure on BAL functionality.

For this study, the HepaRG-AMC-BALs that had been exposed to ALF plasma from t = 1 hour to t = 6 hours were designated the ‘mild ALF’ group, and the HepaRG-AMC-BALs that had been exposed to ALF plasma from t = 6 hours until death form the ‘severe ALF’ group. As a control group HepaRG-AMC-BALs were tested before connection to rats.
Composition of ALF plasma
During the animal experiments, rat plasma samples were drawn at 0, 6 and 14 hours after total liver ischemia from the extracorporeal BAL system at the carotid artery line. To confirm ALF, these samples were investigated for concentrations of ammonia and creatinine, and activities of aspartate aminotransferase (AST) and lactate dehydrogenase (LDH), as described.\(^3\)

Quantitative reverse transcription-polymerase chain reaction
We analyzed gene expression levels of samples of the BAL matrix containing cells (T-bags) harvested from HepaRG-AMC-BALs (control, mild ALF and severe ALF groups, n = 5) as described.\(^{22, 23}\) Transcript levels were calculated, subsequently normalized for 18S ribosomal RNA and expressed as a percentage of the mean of two frozen human liver samples.\(^{24}\) These samples derived from two female patients, aged 40 and 41 years, with liver adenoma and no elevated liver damage, history of medication or alcohol abuse. The procedure was in accordance with the ethical standards of the institutional committee on human experimentation and the Helsinki Declaration of 1975.

BAL function and cell damage parameters
The HepaRG-AMC-BALs were tested before (control, n = 6), and immediately after ALF-plasma exposure (mild ALF, n = 5; severe ALF, n = 3). To this end, HepaRG-AMC-BALs were first flushed with 30 mL test medium (HepaRG culture medium supplemented with 125 µM testosterone (Sigma Aldrich), 1.5 mM \(^{15}\)NH₄Cl (Sigma Aldrich), 2 mM L-lactate (Sigma Aldrich), and 2.75 mM D-galactose (Sigma Aldrich)), followed by a 24-hour period of recirculation with 100 mL of test medium. Samples (1 mL) were taken at 0.5, 1, 2, 8 and 24 hours of recirculation and analyzed for concentrations of ammonia, urea, \(^{15}\)N-urea, 6β-hydroxytestosterone (6β-OHT), apolipoprotein A-1, amino acids, and activities of AST and LDH as described.\(^{22, 25-27}\) Function and damage parameter rates were determined by calculating the changes in concentration/activity in medium per hour per BAL over a testing period with linear change in time. The rate of 6β-OHT production (CYP3A4 activity) and ammonia elimination were calculated in the first 30 minutes and 2 hours of testing, respectively. Production rates of urea, \(^{15}\)N-urea, apolipoprotein A-1, and amino acids, as well as AST and LDH leakage, were calculated over 24 hours of testing.

Statistical analysis
We compared differences between two experimental groups using unpaired Student’s t-tests. SPSS 16.0.1 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Prism version 4.01 (GraphPad Prism Inc, San Diego, CA, USA) was used for graphical presentation of the data. Data are expressed as means ± standard deviations. Significance was reached if \(p < 0.05\).
RESULTS

Composition of ALF plasma during mild- and severe-ALF-plasma exposure
We analyzed the composition of the rat ALF plasma during HepaRG-AMC-BAL treatment at t = 0, 6, and 14 hours after induction of ALF (Table 1). Notably, the average concentration of ammonia at t = 0 was already above reference values, due to the existing porto-caval shunt as from 3 days prior to the total liver ischemia.28,29 The ammonia and creatinine levels continuously increased during the treatment (3.5- and 3-fold from t = 0 to 14 h, respectively), whereas the AST and ALT levels increased during the first 6 hours (219- and 174-fold, respectively) and then remained high until finalization of the experiment. During the experiments, all rats developed clinical signs of ALF and eventually died between 15 and 16 hours after onset of complete liver ischemia.3

Table 1. The composition of the ALF-rat plasma during treatment with the HepaRG-AMC-BALs.

<table>
<thead>
<tr>
<th>Time after induction of ALF (h)</th>
<th>Ammonia (µM)</th>
<th>Creatinine (µM)</th>
<th>ALT (U/L)</th>
<th>AST (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>t = 0</td>
<td>283 ± 4</td>
<td>27 ± 5</td>
<td>75 ± 20</td>
<td>144 ± 12</td>
</tr>
<tr>
<td>t = 6</td>
<td>782 ± 26</td>
<td>32 ± 6</td>
<td>16425 ± 4441</td>
<td>24996 ± 2298</td>
</tr>
<tr>
<td>t = 14</td>
<td>992 ± 185</td>
<td>74 ± 27</td>
<td>14978 ± 4392</td>
<td>30394 ± 6444</td>
</tr>
<tr>
<td>reference values (rat)</td>
<td>27 ± 10</td>
<td>48 - 53</td>
<td>49 - 67</td>
<td>96 - 153</td>
</tr>
</tbody>
</table>

Abbreviations: ALF, acute liver failure; AMC-BAL, Academic Medical Center-bioartificial liver; ALT, alanine aminotransferase; AST, aspartate aminotransferase. Values are average concentrations ± standard deviations, or in case of intervals, 2.5th - 97.5th percentiles.

ALF-Plasma Exposure Marginally Increases Cell Damage in the HepaRG-AMC-BAL
To assess the effect of ALF plasma to the HepaRG-AMC-BALs, we compared the HepaRG-AMC-BALs not exposed to any plasma (control group) with those either used for treatment during the first 5 hours after induction of ischemia (mild ALF group), or during the following 10 hours (severe ALF group). AST leakage increased 2.3- and 2.9-fold, in mild and severe ALF groups, respectively, relative to the control group (Fig 1A). In contrast, LDH leakage was not increased in both ALF groups, although we observed a trend towards an increase in the severe ALF group (Fig. 1B).

Notably, the observed leakage in 24 hours in the control group corresponds with only 2-3% of their total cellular contents (data not shown), implicating a limited increase in cell damage in the ALF-plasma-exposed groups.3
Effects of acute-liver-failure-plasma exposure on hepatic functionality of HepaRG-AMC-Bioartificial Liver

Figure 1. The effect of ALF plasma on cell damage of the HepaRG-AMC-BAL as measured by AST leakage (A) and LDH leakage (B). Abbreviations: ALF, acute liver failure; AMC-BAL, Academic Medical Center Bioartificial Liver; AST, aspartate transaminase; LDH, lactate dehydrogenase. Values are expressed as means ± standard deviations. Significance: \( p < 0.05 \) versus control group. No significant differences between mild and severe ALF groups.

ALF-plasma exposure decreases transcript levels of various hepatic genes in the HepaRG-AMC-BAL

Both mild and severe ALF groups contained 70-80% lower transcript levels of the hepatic transcription factors HNF4\( \alpha \) (hepatic nuclear factor 4\( \alpha \); nuclear receptor subfamily 2, group A, member 1) and PXR (pregnane X receptor; nuclear receptor subfamily 1, group I, member 2), where the control group levels were comparable to human liver (Fig 2A, B).

Figure 2. The effect of ALF plasma on transcript levels of hepatic transcription factors HNF4\( \alpha \) (A) and PXR (B) of HepaRG-AMC-BALs. Abbreviations: ALF, acute liver failure; AMC-BAL, Academic Medical Center Bioartificial Liver; HNF4\( \alpha \), hepatic nuclear factor 4\( \alpha \); PXR, pregnane X receptor. Values are expressed as means ± standard deviations. Significance: \( p < 0.05 \) versus control group. No significant differences between mild and severe ALF groups.
Transcript levels of the urea cycle enzymes carbamoyl phosphate synthetase and arginase 1 did both not significantly differ in the ALF groups compared to the control group, although there was a trend towards a decrease for both (Fig 3A, B). In contrast, the transcript level of the non-urea cycle associated enzyme arginase 2 increased 3-fold in the severe ALF group (Fig 3C).

We found a 2-fold decrease in the expression of glutamine synthetase in the severe ALF group, and no change in the mild ALF group (Fig 3D). Nonetheless, expression of glutamine synthetase was still high after severe-ALF-plasma exposure, being approximately 2-fold the expression level in human liver. Glutamine synthetase catalyzes the production of glutamine from glutamate and ammonia, and thereby provides an alternative route to eliminate ammonia in addition to the urea cycle.

**Figure 3.** The effect of ALF plasma on transcript levels of hepatic genes involved in ammonia elimination and/or urea production of HepaRG-AMC-BALs: CPS (A), ARG1 (B), ARG2 (C), and GS (D). Abbreviations: ALF, acute liver failure; AMC-BAL, Academic Medical Center Bioartificial Liver; ARG1, arginase 1; CPS, carbamoylphosphate synthetase; GS, glutamine synthetase; ARG2, arginase 2. Values are expressed as means ± standard deviations. Significance: \(^{a}p < 0.05\) versus control group. No significant differences between mild and severe ALF groups.
The effects of ALF plasma on the transcript levels of various CYP genes were more evident. Transcript levels of CYP1A2, CYP2B6, and CYP3A4 decreased 6-, 40- and 3-fold, respectively, and for CYP3A7 expression we also observed a trend towards a decrease (Fig 4).

In conclusion, except for the non-hepatic gene arginase 2, ALF-plasma exposure decreased the transcript levels of two important hepatic transcription factors and most of the tested hepatic genes, and the decrease was most pronounced upon severe-ALF-plasma exposure.

**Figure 4.** The effect of ALF plasma on transcript levels of hepatic enzymes involved in drug metabolism of HepaRG-AMC-BALs: CYP1A2 (A), CYP2B6 (B), CYP3A4 (C), CYP3A7 (D). Abbreviations: ALF, acute liver failure; AMC-BAL, Academic Medical Center Bioartificial Liver; CYP, cytochrome p450. Values are expressed as means ± standard deviations. Significance: $p < 0.05$ versus control group. No significant differences between mild and severe ALF groups.

**ALF-plasma exposure has mainly positive effects of on hepatic functionality of the HepaRG-AMC-BAL**

Whereas the transcript levels of many hepatic genes decreased substantially upon ALF-plasma exposure, most of the tested hepatic functions remarkably remained unchanged or increased. For example, where the transcription level of glutamine synthetase decreased more than 2-fold (Fig 3D), the rate of ammonia elimination was unaffected (Fig 5A). As glutamine synthetase
activity probably accounts for the bulk of the ammonia elimination in the HepaRG-AMC-BAL, this implies a differential effect of ALF plasma on transcript level compared to activity level of the critical enzyme.3

A similar discrepancy between transcript level and enzymatic activity was found for CYP3A4, as its activity (measured by the 6β-hydroxylation of testosterone) was increased 1.5-fold in the severe ALF group compared to the control group (Fig. 5B) while its transcript level was 3-fold decreased (Fig 4C).

Furthermore, the total urea production was 1.5-fold increased in the severe ALF group relative to the control group (Fig. 5C). Yet, the conversion rate of 15N ammonia into 15N urea, was unchanged, and even decreased 2-fold in the mild ALF group (Fig 5D) which may therefore have been a temporary effect.

Finally, the apolipoprotein A-1 production in the severe ALF group was 1.5-fold the rate of the control group (Fig 5E). Notably, intracellular levels of apolipoprotein A-1 were negligible (data not shown), excluding the possibility of leakage as a cause for the observed increase.

In conclusion, in our setup, severe-ALF-plasma exposure resulted in a significant increase of most hepatic functions assessed, while mild-ALF-plasma exposure did not significantly affect HepaRG-AMC-BAL functionality, except for a possibly temporary decreased conversion of ammonia into urea.

ALF-Plasma exposure increases amino acid consumption
To further investigate the effects of ALF-plasma exposure on ammonia metabolism, we analyzed the amino acid metabolism of the HepaRG-AMC-BALs. The control BAL group, consumed amino acids, and this consumption was further increased 2-fold in the mild ALF and severe ALF groups (Table 2) up to a level of 16.8 µmol/h. Of note, some amino acids were almost depleted after 24 hours of testing in the ALF-plasma-exposed groups, and therefore the calculated effect of ALF-plasma exposure on the metabolism of these amino acids may even be an underestimation of the actual effect. The increased consumption of alanine, leucine, and isoleucine (2.7-, 1.7-, and 1.8-fold, respectively) contributed primarily to this increase in total amino acid consumption in the severe ALF group. The only amino acids that were net produced were glutamine and ornithine, and their production rates were not significantly affected by ALF-plasma exposure, although there was a trend towards increased production. The unchanged glutamine metabolism after ALF-plasma exposure is in accordance with the unchanged ammonia elimination after ALF-plasma exposure. Of the amino acids involved in the urea cycle, only arginine consumption that were net produced were glutamine and ornithine, and their production rates were not significantly affected by ALF-plasma exposure, although there was a trend towards increased production. The unchanged glutamine metabolism after ALF-plasma exposure is in accordance with the unchanged ammonia elimination after ALF-plasma exposure. Of the amino acids involved in the urea cycle, only arginine consumption increased significantly 2.5-fold in the severe ALF group compared to the control group, which is in line with the observed increase in urea production (Fig. 5C) and arginase 2 transcript levels (Fig. 3C), without a concurrent increase in carbamoyl phosphate synthetase transcript levels (Fig. 3A). This indicates that the increased urea production can be attributed to arginase 2 activity, and does not reflect increased conversion of ammonia into urea.
Figure 5. The effect of ALF plasma on hepatic functions of HepaRG-AMC-BALs as measured by ammonia elimination (A), 6β-OHT production (B), urea production (C), 15N-urea production (D), and APOA1 production (E). Abbreviations: ALF, acute liver failure; AMC-BAL, Academic Medical Center Bioartificial Liver; 6β-OHT, 6β-hydroxytestosterone; APOA1, apolipoprotein A-1. Values are expressed as means ± standard deviations. Significance: *p < 0.05 versus control group, †p < 0.05 versus mild ALF group.
## Table 2. Amino acid metabolism in control and ALF-plasma exposed HepaRG-AMC-BALs.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Control (µmol/h)</th>
<th>Mild ALF (µmol/h)</th>
<th>Severe ALF (µmol/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALA</td>
<td>-0.95 ± 0.41</td>
<td>-2.64 ± 0.87 **</td>
<td>-2.61 ± 1.79</td>
</tr>
<tr>
<td>ARG *</td>
<td>-0.60 ± 0.21</td>
<td>-1.16 ± 0.60</td>
<td>-1.48 ± 0.39 ^</td>
</tr>
<tr>
<td>ASN</td>
<td>-0.13 ± 0.07</td>
<td>-0.17 ± 0.21</td>
<td>-0.29 ± 0.06 ^</td>
</tr>
<tr>
<td>ASP *</td>
<td>N/A</td>
<td>-1.12 ± 0.32</td>
<td>-1.41 ± 0.39</td>
</tr>
<tr>
<td>CIT</td>
<td>-0.04 ± 0.10</td>
<td>-0.01 ± 0.01</td>
<td>-0.03 ± 0.01</td>
</tr>
<tr>
<td>GLN</td>
<td>1.58 ± 0.47</td>
<td>0.82 ± 1.35</td>
<td>2.34 ± 0.48</td>
</tr>
<tr>
<td>GLU *</td>
<td>-1.16 ± 0.35</td>
<td>-1.15 ± 0.53</td>
<td>-1.76 ± 0.06 ^</td>
</tr>
<tr>
<td>GLY</td>
<td>N/A</td>
<td>-0.81 ± 0.66</td>
<td>-1.19 ± 0.78</td>
</tr>
<tr>
<td>HIS</td>
<td>-0.29 ± 0.12</td>
<td>-0.30 ± 0.12</td>
<td>-0.39 ± 0.12</td>
</tr>
<tr>
<td>ILE</td>
<td>-1.11 ± 0.33</td>
<td>-1.61 ± 0.51</td>
<td>-2.04 ± 0.09 ^</td>
</tr>
<tr>
<td>LEU *</td>
<td>-1.74 ± 0.42</td>
<td>-2.51 ± 1.04</td>
<td>-3.04 ± 0.16 ^</td>
</tr>
<tr>
<td>LYS</td>
<td>-1.51 ± 0.75</td>
<td>-1.05 ± 0.24</td>
<td>-1.35 ± 0.08</td>
</tr>
<tr>
<td>MET *</td>
<td>-0.27 ± 0.09</td>
<td>-0.40 ± 0.20</td>
<td>-0.45 ± 0.03 ^</td>
</tr>
<tr>
<td>ORN</td>
<td>0.28 ± 0.21</td>
<td>0.46 ± 0.32</td>
<td>0.64 ± 0.27</td>
</tr>
<tr>
<td>PHE *</td>
<td>-0.20 ± 0.07</td>
<td>-0.25 ± 0.11</td>
<td>-0.30 ± 0.03 ^</td>
</tr>
<tr>
<td>SER</td>
<td>-0.19 ± 1.16</td>
<td>-0.12 ± 0.22</td>
<td>-0.35 ± 0.41</td>
</tr>
<tr>
<td>TAU</td>
<td>-0.44 ± 0.17</td>
<td>-0.91 ± 0.26 ^</td>
<td>-1.04 ± 0.11 ^</td>
</tr>
<tr>
<td>THR</td>
<td>-0.46 ± 0.19</td>
<td>-0.87 ± 0.23 ^</td>
<td>-1.09 ± 0.08 ^</td>
</tr>
<tr>
<td>TRP</td>
<td>-0.42 ± 0.22</td>
<td>-0.72 ± 0.20</td>
<td>-0.80 ± 0.03 ^</td>
</tr>
<tr>
<td>TYR</td>
<td>-0.01 ± 0.01</td>
<td>-0.02 ± 0.01</td>
<td>-0.01 ± 0.01</td>
</tr>
<tr>
<td>VAL</td>
<td>-0.82 ± 0.26</td>
<td>-1.19 ± 0.31</td>
<td>-1.53 ± 0.12 ^</td>
</tr>
<tr>
<td>Total</td>
<td>-8.49 ± 5.62</td>
<td>-14.61 ± 7.99 ^</td>
<td>-16.77 ± 5.11 ^</td>
</tr>
</tbody>
</table>

Amino acid production and consumption are indicated as positive and negative values, respectively. * Indicates amino acids with almost depleted levels (in both ALF-plasma exposure groups) after 24 hours of testing, and their calculated consumption may therefore be an underestimation of the actual consumption. Abbreviations: AMC-BAL, Academic Medical Center-bioartificial liver; ALF, acute liver failure; N/A, not available. Values are given as averages ± standard deviations (n = 3 to 5). ^p < 0.05 versus control group. No significant differences were observed between mild and severe ALF groups.

## DISCUSSION

The HepaRG-AMC-BAL has recently demonstrated efficacy in a rat model of ALF. However, for clinical application, it is essential that its functionality is preserved sufficiently during treatment, and therefore it should resist the toxic components of ALF in a background of the patient's plasma. Here, we show that 10 hours exposure to severe ALF plasma did not affect hepatic functionality of the HepaRG-AMC-BAL, but remarkably, increased several hepatic functions. Specifically, the production of 6β-OHT, apolipoprotein A-1, and total urea increased 1.5- to 2-fold, whereas the elimination of ammonia and the conversion of ammonia into urea remained relatively unchanged. In addition, ALF-plasma exposure only mildly increased cell damage as measured by AST and LDH release. In contrast, transcript levels of most hepatic genes decreased already upon mild-ALF-plasma exposure for 5 hours up to maximally 40-fold.
This observed discrepancy in effects on hepatic functionality and the gene transcript levels may be explained by differences in the stability of mRNAs versus proteins. Transcript RNA is a relatively unstable molecule with a mean half-life of 10 to 20 hours under normal conditions, while the half-life of proteins is in the order of days. Therefore, an ALF-plasma-exposure-induced downregulation of gene transcription may lead to a decrease in transcript levels within hours, whereas a decline in protein activity will occur substantially later. In addition, post-transcriptional regulation may increase the activity of detoxification related proteins, to reduce the increased toxin concentrations that occur during ALF.

As samples for transcript level analyses were taken directly after ALF-plasma exposure and functions are assessed in culture medium over a period of 24 hours after ALF-plasma exposure, one might also postulate that recovery of the cells may underlie the difference between the observed decrease in transcript levels and the concurrent increase in functionality. However, this is unlikely, as the concentrations of all products and substrates of the assessed hepatic functions showed a linear change in the period tested.

The rate of urea production increased 1.5-fold while the rate of $^{15}$N-urea production decreased 2-fold or remained stable upon mild-ALF-plasma or severe-ALF-plasma exposure, respectively. The rate of $^{15}$N-urea production is a measure for the conversion of ammonia into urea, and largely reflects urea cycle activity. Interestingly and similar to our findings in monolayer cultures of HepaRG cells, the total urea production in HepaRG-AMC-BALs increased upon ALF-plasma exposure. Probably, this increase in urea production is explained by an increase in arginase 2 activity, as the conversion of ammonia into urea did not change and arginase 2 expression, arginine consumption, and ornithine production increased after exposure to ALF plasma. Although the exact function of arginase 2 in hepatocytes is unclear, it is noteworthy that arginase 2 is upregulated by lipopolysaccharides and cytokines as part of the inflammatory response in macrophages and endothelial cells. As ALF induces generalized inflammation, a similar mechanism may underlie the observed increase in arginase 2 expression in HepaRG-AMC-BALs after ALF-plasma exposure.

We also observed an increase in apolipoprotein A-1 production upon ALF-plasma exposure, which might prove beneficial for ALF patients, as apolipoprotein A-1 exerts anti-inflammatory activity. Nonetheless, it is a remarkable finding, as apolipoprotein A-1 expression is suppressed by cholate and inflammatory cytokines, which are abundantly present in ALF plasma.

In conclusion, the finding that exposure to mild ALF plasma for 5 hours did not affect most hepatic functions of the HepaRG-AMC-BAL and that exposure to severe ALF plasma for 10 hours even resulted in a slight increase of its overall hepatic functionality holds promise for clinical application of this BAL. In this respect, it is also noteworthy that we used rat-derived ALF plasma, and therefore, an additional xenogenic effect – that is absent when treating patients – cannot be excluded. Nonetheless, further research on the effects of human ALF plasma on the HepaRG-AMC-BAL is necessary to obtain an indication of the life-time of a HepaRG-AMC-BAL during treatment of ALF patients.
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