Human liver cell lines for the AMC-bioartificial liver

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CURRICULUM VITAE

Born on January 6th 1978 in Boxtel, the Netherlands, Gerardus (Geert) Adrianus Antonius Nibourg grew up as the elder brother of two sisters. After graduating from the Gymnasium at the Jacob Roelands Lyceum in Boxtel in 1996, he started his medical training at the University of Leuven, Belgium. After one year, he continued his studies at the University of Utrecht, the Netherlands. As a medical student he enjoyed an optional clinical elective in South Africa, at the Tygerberg Hospital, University of Stellenbosch, Cape Town. His enthusiasm for scientific research translated in the participation in optional research projects at the departments of Experimental Nephrology (Prof. dr. M.C. Verhaar) and Surgical Oncology (Prof. dr. I.H.M. Borel Rinkes) at the University of Utrecht during his studies. To gain clinical experience, he started working as an emergency physician at the University Medical Center, Utrecht, after he had graduated from medical school in 2005. After this year, he started a PhD program at the Academic Medical Center (AMC), Amsterdam. The program comprised the development of a suitable human biocomponent for the AMC Bioartificial Liver under the supervision of dr. R.A.F.M. Chamuleau, dr. R. Hoekstra and Prof. dr. T.M. van Gulik, a combined effort of the Surgical Laboratory and the Tytgat Institute for Liver and Intestinal Research. This program resulted in the present thesis. The paper 'Liver progenitor cell line HepaRG differentiated in a bioartificial liver effectively supplies liver support to rats with acute liver failure.' (Chapter 6) was awarded with the best paper prize at the 9th congress of the European-African Hepato-Pancreato-Biliary Association in Cape Town, South Africa in 2011. The research also led to filing of a patent on differentiated human liver cell cultures and their use in bioartificial liver systems. In April 2011, he started a two-year residency in Internal Medicine (dr. P.H.J. Frissen and dr. Y.F.C. Smets) at the Onze Lieve Vrouwe Gasthuis in Amsterdam, as part of a six-year training program in Gastroenterology and Hepatology (Prof. dr. P. Fockens and Prof. dr. U.H.W. Beuers) at the AMC in Amsterdam. He lives together with his girlfriend Iris van Dijk in Amsterdam, and in his spare time, he loves to surf, read, play the piano, travel, and play field hockey.

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2012

COLOPHON

The studies described in this thesis were performed at the Department of Surgery and the Tytgat Institute for Liver and Intestinal Research, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands.

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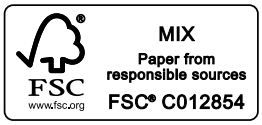

Human liver cell lines for the AMC-bioartificial liver

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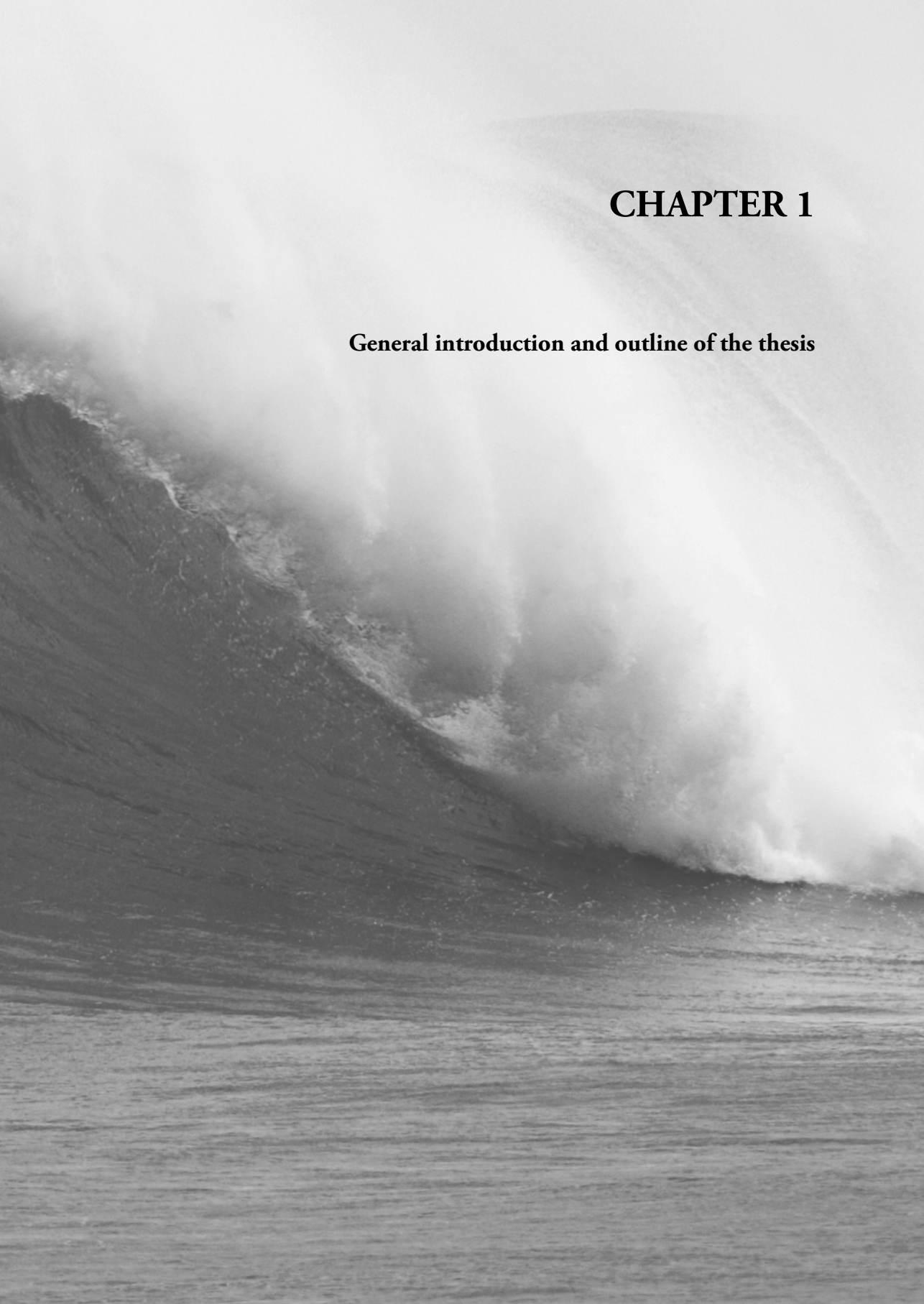

CHAPTER 1

General introduction and outline of the thesis

GENERAL INTRODUCTION

Severe liver failure (SLF), caused by loss of more than 60% functional liver mass, is a devastating syndrome with a high morbidity and mortality. SLF comprises two different syndromes: acute liver failure (ALF) and acute-on-chronic liver failure (ACLF). For ALF more than 40 different definitions can be found in literature.[1] A common feature of all definitions is the development of hepatic encephalopathy (HE) within a number of weeks (2 to 26) after the onset of liver disease in a person with a previously normal liver. In contrast, ACLF is defined as an acute deterioration of liver function, including the development of HE, in a patient with pre-existing liver disease.

The etiology of ALF is diverse and incidences differ per geographic region, but is most commonly related to acute viral hepatitis (most frequently hepatitis A, B, and E), drug overdose (most frequently acetaminophen), and idiosyncratic drug reactions. In contrast, in the case of ACLF previously well-compensated liver disease derails by an acute insult such as variceal bleeding, a bacterial infection, or dehydration.

Pathologically, ALF and ACLF are both characterized by massive hepatocellular necrosis and/or apoptosis, in the case of ACLF often superimposed on cirrhosis. Clinically, both ALF and ACLF give rise to a number of severe and life-threatening complications, such as coagulopathy, hepatic encephalopathy, systemic vasodilatation, metabolic acidosis, uncontrolled sepsis, and finally multi-organ failure.[2]

Although the pathophysiological events that occur and give rise to these complications in both syndromes are immensely complex and still not fully understood, a number of sequelae has been described.[3] In the case of ALF, these pathophysiological processes can grossly be divided into three major sequelae. The first is the attenuation of hepatic protein synthesis, which leads to coagulopathy and alterations in plasma and brain amino acid composition, *e.g.* an increase in aromatic amino acids and a decrease in branched chain amino acids. The second is the reduced clearance of endogenous and exogenous toxins, such as ammonia, lactate, mercaptans, indoles, and benzodiazepine-like substances, which plays an important role in the development of HE, renal failure, and metabolic acidosis.[4, 5] Of note, astrocyte swelling in the brain is a key event in the development of HE, and hyperammonemia plays an important role in astrocyte swelling. Ammonia cannot efficiently be removed from the circulation by the synthesis of urea in the liver, but ammonia can still be incorporated into glutamine in muscle and brain. as an alternative route for ammonia detoxification. Interestingly however, there is accumulating evidence that the temporary fixation into glutamine is a crucial step in the pathogenesis of astrocyte swelling (Trojan Horse hypothesis). [6] Glutamine is taken up by the astrocyte's mitochondrion, and subsequent hydrolysis of through the activity of the enzyme glutaminase yields high levels of ammonia again. Here, ammonia induces oxidative stress that results in mitochondrial swelling and dysfunction, which leads to alterations in energy metabolism, signalling mechanisms,

astrocytic glutamate uptake, and cell swelling. The third major pathophysiological sequela in ALF is the development of immune dysfunction in which elevated systemic endotoxin levels play an important causative role. There is accumulating evidence that this is initiated by increased intestinal leakage, reduced Kupffer cell mass, and portal-hypertension induced portosystemic shunting. In addition, release of intracellular debris (DNA, RNA, and cytoskeletal fragments) from the necrotic liver into the circulation elicits an inflammatory reaction. The endotoxins and cellular fragments induce the release of various pro-inflammatory cytokines that fuel systemic inflammation and induce a release in endothelial-derived nitric oxide, the latter resulting in vasodilatation and a hyperdynamic circulation.[7] In ACLF, the interaction between immune dysfunction, bacterial translocation from the gut, and circulatory dysfunction are the most important pathophysiological events.[8]

Despite the progress made in supportive care, only 20% of ALF and ACLF patients survive without liver transplantation, which increases the survival rates to over 80%[9, 10] However, liver transplantation is limited by the scarcity of donor organs. In the US, about 20% of the patients with severe liver failure who are on the waiting list for liver transplantation die while waiting for a donor liver.[11] Moreover, improving the patient's condition before liver transplantation substantially increases post-transplant survival.[12] Lastly, temporary liver support could potentially create time for the diseased liver to regenerate, thereby rendering liver transplantation superfluous. Taken together, there is an urgent need for liver support therapy.

Liver support systems can be divided into non-biological (artificial) and biological (bioartificial) systems. Non-biological systems typically use adsorbents and/or dialyzing membranes to detoxify the patients' plasma, but have not yet shown a clear effect on survival of ALF and ACLF patients.[13-15] Probably the potential of these systems is limited by their lack of metabolizing and synthesizing capacity, as well as the fact that detoxification in these systems is non-specific, which might lead to the removal of beneficial proteins, such as growth factors and clotting factors.[16]

In contrast to non-biological systems, bioartificial livers (BALs) typically comprise an extracorporeal bioreactor that is loaded with a biocomponent, *e.g.* hepatocytes, that can be perfused with the patient's blood or plasma. These systems can thereby potentially replace the majority of the liver's many functions.[17] To date, BAL devices with various configurations, biocomponents and culture strategies have been developed.[18]

The AMC-BAL that was developed in the AMC is based on a bioreactor with an internal oxygenator and a spirally wound, non-woven polyester matrix, yielding a three-dimensional culture environment that provides direct cell-plasma contact and equal and optimal oxygenation of the biocomponent (Fig. 1 and 2).[19]

Primary human hepatocytes are of course the ideal cell source for a BAL, but their availability is limited. Alternatively, BALs based on primary porcine hepatocytes have proven efficacious in animal models of ALF.[20, 21] However, the use of animal-derived cells is compromised by

xenotransplantation-related risks. Therefore, there is an urgent need for a human biocomponent with sufficient hepatic functionality that is also available in large quantities. In addition, the safety of this biocomponent should be warranted, which implies the absence of pathogens and preferably also of tumorigenicity.

The main objective of this thesis was to develop a BAL based on a human biocomponent with characteristics as indicated above, and to test its efficacy in an animal model of ALF.

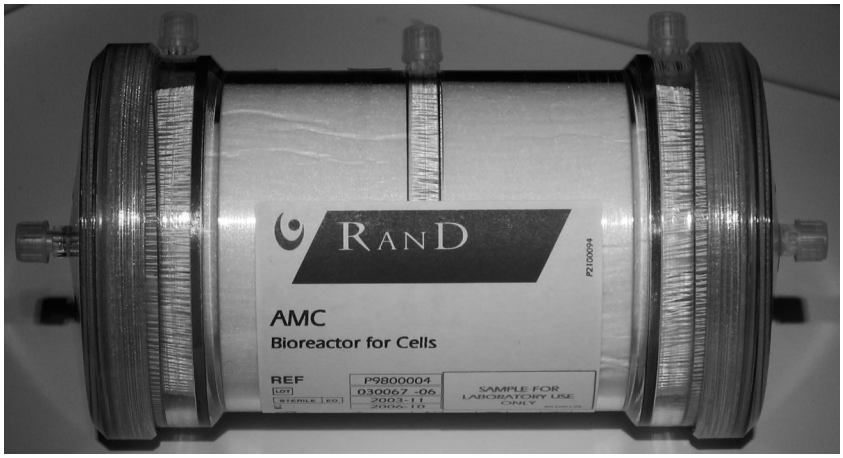

Figure 1. Photograph of the AMC-BAL. *For color figure, see page 211.*

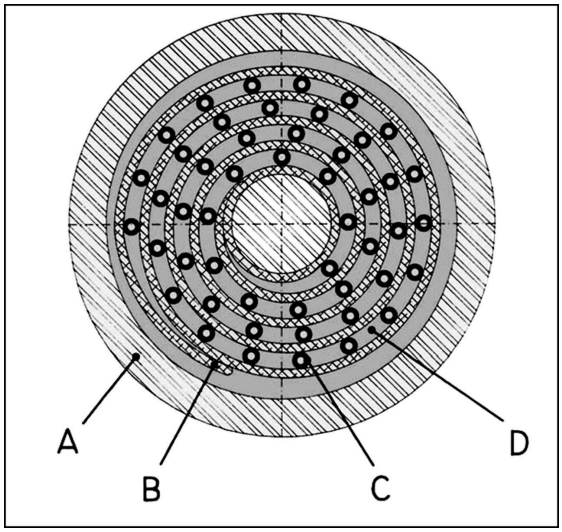

Figure 2. Schematic cross section of the AMC-BAL showing the polycarbonate housing (A), the spirally wound non-woven polyester matrix (B), the hollow semi-permeable oxygenation capillaries (C), and the extra-capillary space (D) through which the culture medium or patients plasma is perfused.

OUTLINE OF THE THESIS

Firstly, in **Chapter 2,** we reviewed all proliferative human cell sources that have been applied in BAL systems so far. We analyzed their potential for clinical BAL application by comparing their *in vitro* hepatic functionality with primary hepatocytes and discussed their efficacy in animal models of acute liver failure and/or their clinical efficacy as assessed in patient studies.

In **Chapter 3**, we described the development and *in vitro* evaluation of the novel cell line cBAL119, that was generated by overexpressing the *pregnane X receptor* gene, a master regulator of detoxification, in the human hepatoma cell line HepG2. We assessed its potential for BAL application by studying its hepatic functionality cultured on monolayer as well as in a laboratory model of the AMC-BAL.

Chapter 4 describes the potential of another human hepatoma cell line for BAL application: the HepaRG cell line. This bipotent liver progenitor cell line differentiates *in vitro* into two distinct types of cells upon reaching confluence in monolayer cultures: hepatocyte-like cells and biliary-like cells. Subsequent culturing in the presence of 2% dimethyl sulfoxide (DMSO) further increases its detoxification function, but other hepatic functions are largely unknown. In this chapter, the HepaRG cells have been cultured on monolayer using different cell culture protocols – with and without DMSO. We studied transcript levels of various hepatic genes, cell damage parameters, and several liver-specific functions.

In **Chapter 5** we cultured the HepaRG cells in a laboratory scale AMC-BAL, also with and without DMSO, and studied the morphology of the HepaRG cells, transcript levels of various hepatic genes, and a number of hepatic functional parameters. Subsequently, we tested if treatment with this HepaRG-AMC-BAL could increase the survival time of rats with ALF.

As opposed to a static monolayer culture that relies on passive diffusion for oxygenation, cell culture in the AMC-BAL is three-dimensional, actively oxygenated, and continuously perfused. In **Chapter 6**, we studied whether, for HepaRG cells, these differences in culture environment translate in differences in morphology, gene expression, and hepatic functionality. In addition, the effect of BAL culture time on these parameters was studied.

During treatment of ALF patients, the biocomponent of a BAL is exposed to ALF plasma that contains various toxic metabolites. Prolonged exposure to ALF plasma may therefore negatively affect the functionality of a BAL. Firstly, in **Chapter 7**, we tested this hypothesis on monolayer cultures of HepaRG cells by exposing them to rat derived ALF plasma and by studying the effects histologically, on cell damage parameters, on transcript levels of hepatic genes, and on several hepatic functions. In **Chapter 8**, this hypothesis was tested again, but this time on the HepaRG-AMC-BAL. Essentially the same parameters were studied, but in this study we additionally studied the effects of exposure to plasma of rats with both mild and severe ALF, *i.e.* 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).

In **Chapter 9**, we assessed the optimal medium perfusion flow rate for the HepaRG-AMC-BAL. To this end we perfused laboratory scale HepaRG-AMC-BALs with culture medium at four different perfusion flow rates and studied the effects on hepatic functionality and cell damage. In addition, we calculated mass balances of ammonia and lactate over the HepaRG-AMC-BAL with the purpose of evaluating their potential in monitoring BAL condition during the treatment of ALF patients.

The HepaRG cell line has been recognized as a promising source for *in vitro* testing of the metabolism and toxicity of drugs and other compounds. However, the hepatic differentiation of these cells has so far relied on exposure to DMSO, which has a damaging effect and represses all-round hepatic functionality. In **Chapter 10** therefore, the potential of the HepaRG-AMC-BAL cultured without DMSO was evaluated for testing the metabolism and toxicity of drugs and compounds. To this end, these HepaRG-AMC-BALs were tested for phase 1 and phase 2 drug metabolism, as well as bile acid metabolism.

Lastly, **Chapter 11** summarizes the studies of this thesis and discusses future studies.

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CHAPTER 2

Proliferative human cell sources applied as biocomponent in bioartificial livers: a review

Expert Opinion on Biological Therapy 2011;43(10):1483-1489.

Geert A.A. Nibourg
Robert A.F.M. Chamuleau
Thomas M. van Gulik
Ruurdtje Hoekstra

ABSTRACT

Background: Bioartificial livers (BALs) are urgently needed to bridge severe liver failure patients to liver transplantation or liver regeneration. When based on primary hepatocytes, their efficacy has been shown in animal experiments and their safety was confirmed in clinical trials. However, a proliferative human cell source with therapeutic functionality is needed to secure availability and move BAL application forward

Aim: This review compares the performance of BALs based on proliferative human biocomponents and on primary hepatocytes.

Methods: This review evaluates relevant studies identified by searching the MED-LINE database until July 2011 and some of our own unpublished data.

Results and conclusions: All the discussed hepatocyte-like biocomponents show deficiencies in their hepatic functionality compared to primary hepatocytes, particularly functions occurring late in liver development. Nonetheless, the HepaRG, HepG2-GS-CYP3A4 and mesenchymal stem cells show efficacy in a statistically well-powered animal model of acute liver failure, when applied in a BAL device. Various methods to gain higher functionality of BALs, including genetic modification, the usage of combinatory cell sources, and improvement of culture methods have scarcely been applied, but may further pave the path for BAL application. Clinical implementation of a BAL based on a human proliferative biocomponent is still several years away.

INTRODUCTION

Acute liver failure (ALF) and acute-on-chronic liver failure (ACLF) are severe syndromes with mortality rates as high as 80%.[1] In this review the term severe liver failure (SLF) will be used for both ALF and ACLF. Clinically, the syndromes present as a severe impairment of liver function with hepatocellular necrosis, leading to hepatic encephalopathy (HE), systemic inflammation, and multi-organ failure (MOF). Notably, the pathological processes underlying this cascade of events are only partly understood.

Liver transplantation (LT) is often the only cure for SLF patients, increasing the survival rates to over 80%.[2] However, LT is limited by the scarcity of donor organs. In the Western world, about 20-25% of all liver patients (including SLF patients) on the waiting list for LT die while waiting for a donor liver, and this number is increasing. Consequently, there is an urgent need to bridge SLF patients to LT or to liver regeneration. Bioartificial extracorporeal liver support devices can fulfill this need.

Bioartificial livers (BALs) use a biocomponent, *e.g.* hepatocytes in extracorporeal bioreactors that are perfused with the patient's blood or plasma. These systems can theoretically replace all crucial liver functions, and are therefore a more promising option for liver replacement therapy than non-biological systems that only rely on detoxification.[3] BAL devices with various configurations have been developed; Flat plate systems for 2D cultures and systems that support cell organization in 3D relying on semipermeable hollow fibers, scaffolds, and encapsulated cells. All these devices vary in their oxygenation, cell-plasma contact, shear stress, complexity, costs and potential to upscaling.[4-6]

In addition to the device configuration and culture process, successful BAL therapy relies on the hepatic functionality of the biocomponent. There is robust evidence that accumulating levels of ammonia play an important role in the progression of the disease.[1, 7] In addition, several other neurotoxins may act synergistically with ammonia in the progression of HE.[7, 8] Therefore, a BAL should adequately eliminate ammonia and other neurotoxins. Ammonia can be eliminated either by urea production or by fixation into amino acids (predominantly by glutamine synthetase (GS) activity) of which the first route is preferred as urea is an end-product. Other neurotoxins are predominantly detoxified by components associated with drug metabolism, *i.e.* phase 1 (particularly cytochrome P450 (CYP) 3A4) and phase 2 detoxification proteins. In SLF-patients, the lack of other functions such as lactate elimination, glucose homeostasis, and the synthesis of blood proteins including pro- and anti-coagulative factors can lead to serious complications such as acidosis, hypoglycemia, diminished suppression of inflammation, and thrombosis or bleeding.[9-14] Therefore, a BAL should effectively replace these functions as well. In addition, anti-inflammatory properties are probably useful for a BAL, to suppress the systemic inflammation, developing in SLF.[15]

Table 1. Overview of proliferative human cell sources applied in bioartificial livers.

For colored table, see page 212-217.

Tones of grey indicate the potential for BAL application as indicated by in vitro functionality or efficacy in animal studies:
white: no data available.
darkest tone grey: in vitro functionality < 5% of primary hepatocytes, or not effective in animal study;
middle tone grey: in vitro functionality > 10% of primary hepatocytes, or proven efficacy in animal study
lightest tone grey: inconclusive

Biocomponent	Origin	Bioreactor	Tumorgenicity	Growth potential	In vitro functionality	
					Synthesis	Drug-metabolizing activity
	Hepatoma cells					
HepG2	Hepatocellular carcinoma, 15 year old male.	Diverse devices	Pos in immunocomprimised mice *	Immortal	Pos Alb, AFP, ApoA1, fibrinogen	Neg/low in phase I and phase II detoxification
C3A	Clonal derivative of HepG2, selected for strong contact inhibition, high production of albumin and AFP and growth in glucose deficient medium	Diverse devices, most often in ELAD	Neg in immunocomprimised mice	Immortal	Pos Alb, AFP, transferrin	Detectable 7-ethoxycoumarin metabolism, level inconclusive
Huh 7	Hepatoma	Polyurethane foam/sheroid	Pos in immunocomprimised mice	Immortal	Pos Alb	
FlC	Hepatoma	Radial flow		Immortal		
FLC-4	Hepatoma	Radial flow		Immortal	Pos Alb, coagulation factors	Detectable CYP mRNAs, level inconclusive
FLC-5	Hepatoma	Radial flow		Immortal	Pos Alb, coagulation factors	Detectable 6b-hydroxylation, level inconclusive
FLC-7	Hepatoma	Radial flow		Immortal	Pos Alb, coagulation factors	Detectable CYP mRNAs, level inconclusive
HepaRG	Hepatoma	AMC-BAL and multi-compartment hollow fiber capillary membrane	Neg in immunocompromised mice	Immortal	Pos ApoA1	Pos CYP3A4, 6-b hydroxylation testosterone

			Animal studies	Clinical studies	Reference
Urea production**	NH_3 elimination	Carbohydrate metabolism			
Pos, only due to Arg II activity	Inconsistency between reports	Lactate production and glucose consumption	One animal study (n=3) in rat with acetaminophen intoxication using a bioreactor based on alginate incapsulated cells: no effect on survival measured, no effect found on ammonia & bilirubin levels		Nyberg et al. Ann Surg.1994 Yamashita et al. Cell Transplant. 2001 Fukada et al. Cell Transplant. 2003 Rahman et al. Artif Org 2004 Hongo et al. J Biosci Bioeng. 2005 Shimada et al. Hepatogastroenterol. 2007 Coward et al. Art Org. 2009 Nibourg et al. Liver Transplant. 2010
Pos, only due to Arg II activity	Detectable, level inconclusive	Glucose consumption	Only in ELAD. Two inconclusive animal studies (n=3) in anhepatic dogs and dogs with acetaminphen intoxication.	Two clinical phase I studies showing safety. Two randomized controlled clinical trials; the first one not showing positive effects and the second one with unpublished results.	Sussman et al. Hepatol. 1992 Kelly et al. Artif Org. 1992 Sussman et al. Artif Org. 1994 Ellis et al. Hepatol. 1996 David et al. Biotechnol Prog. 2004 Kinasiewicz et al. Transplant Proc. 2007 Kinasiewicz et al. Artif Org. 2008 Kinasiewicz et al. Artif Cells Blood Substit. 2008 Kinasiewicz et al. Int J Artif Org. 2008 Carraro et al. Biomed Microdev. 2008 Harm et al. Int J Artif Org. 2009 Chen et al. Tissue Eng Part A. 2010 Gautier et al. Eur Cells Mat. 2011
					Yamashita et al. Cell Transplant. 2001
					Iwahori et al. Transplant Proc. 2005
No CPS and OTC expression			One animal study (n=2-3) in pigs with α-amanitin and lipopolysaccharide intoxication. Inconclusive results.		Kosuge et al. Liver Int. 2007 Kanai et al. Artif Org. 2007
No CPS and OTC expression					Iwahori et al. Hepatol. 2003 Kosuge et al. Liver Int. 2007
No CPS and OTC expression		Glucose consumption			Kawada et al. In Vitro Cell Dev Biol Animal. 1998 Kosuge et al. Liver Int. 2007
		Lactate consumption, stable glucose levels	One animal study (n=5, 6) in rats with total liver ischemia: positive effect on survival time, HE, bleeding, creatinine and ammonia levels		Darnell et al. Drug Metab Dispos. 2011 Nibourg et al. Unpublished data.

Table 1. Continued

	In vitro immortalized cells					
HepZ	Hepatocyte cell line immortalized through E2F and cyclin D1 overexpression and suppression of pRB and P53	Based on microporous gelatin microcarriers		Immortal		Detectable lidocaine metabolism, level inconclusive
OUMS-29	Fetal hepatocyte cell line immortalized through expression SV40 large and small T antigen	Radial flow		Immortal	Pos Alb	
cBAL111	Fetal hepatocytes immortalized through hTERT overexpression	AMC-BAL		Immortal	Alb level only 6%	
Kobayashi revers imm hTERT PHH	Hepatocytes reversibly immortalized through hTERT overexpression	Hollow fiber		Immortal		
	Fetal cells and stem cells					
Fetal hepatocytes	Fetal liver	AMC-BAL and hollow-fiber		Limited	Pos Alb	Lidocaine metabolism
Small hepatocytes	Liver	Rotary cell culture with cytodex microcarriers		Limited	Relatively low Alb, and substantial decline in time	CYP3A4 protein expression and metabolism of methadone however, no metabolism of morphine and susceptible to toxins
Liver stem cells	Liver	Rotary system		?	Pos Alb	Metabolism 7-ethoxy-4-trifluoromethylcoumarin
Mesenchymal stem cells	Differentiated bone-marrow cells	Flat plate or with 3D nanofibrous scaffold		?	Transferrin, Alb detectable, level inconclusive	Detectable CYP3A4 mRNA, level inconclusive
Differentiated human embryonic stem cells	Blastocyst	Multi-compartment hollow fiber capillary membrane		?	Alb level only <1%	Detectable CYP3A4 and CYP3A7 mRNA, level inconclusive

	Production	Lactate production converting to consumption and glucose consumption			Werner et al. Ann New York Acad Sci. 1999 Werner et al. Biotechnol Bioeng. 2000
					Akiyama et al. Int J Mol Med. 2004
		Lactate production and glucose consumption	One animal study (n=4) in rats with total liver ischemia. No positive effect on survival time.		Poyck et al. J Hepatol. 2008
			Two animal studies. The first study in monkeys with D-galactosamine intoxication with inconclusive results. The second study in pigs with ALF, ongoing.		Kobayashi et al. J Artif Org. 2003 Kobayashi et al. J Hepatobil Pancreat Surg. 2009
	Production	Lactate production and glucose production converting into consumption			Poyck et al. Liver Transpl. 2007 Ring et al. Tissue Eng Part C. 2010 Schmelzer et al. Tissue Eng Part A. 2010
Pos, but substantial decline in time		Lactate production and glucose consumption, but both parameters decline substantially in time			Pavlic et al. Alcohol Clin Exp Res. 2007 Wurm et al. Tissue Eng Part A. 2009 Wurm et al. Tissue Eng Part A. 2010
		Glucose consumption			Fonsato et al. Tissue Eng Part C. 2009
Detectable, level inconclusive			One animal study (n=7) in rats with D-galactosamine intoxication. Positive effects on survival and on leakage of AST and ALT.		Parekkadan et al. PLoS One. 2007 Kazemnejad et al. J Gastroenterol Hepatol. 2009
Detectable, level inconclusive		Lactate production and glucose consumption			Miki et al. Tissue Eng Part C. 2011

Table 1. Continued

	Genetically modified cells					
HepG2-GS	HepG2 with overexpression GS	Radial flow		Immortal		
HepG2-GS-Cyp3A4	HepG2 with overexpression GS and Cyp3A4	Radial flow		Immortal		Detectable diazepam metabolism, level inconclusive
HepG2-DT	HepG2 with overexpression OTC and ArgI	Fluidized bed based on alginate incapsulated cells		Immortal	Pos Alb	
cBAL119	HepG2 with overexpression PXR	AMC-BAL		Immortal	Pos ApoA1	Enhanced 6-b hydroxylation testosterone compared to HepG2, but still low
OUMS-29/H-11	OUMS-29 with overexpression HNF4	Radial flow		Immortal	Pos Alb	CYP3A4 protein present, level inconclusive. Enhanced 6-b hydroxylation testosterone in late culture phase, but still low
TTNT with AdIL-1Ra	Hepatocytes reversibly immortalized through hTERT overexpression, and further expressing IL-1 recepor antagonist	Flat-plate		Immortal		
	Combinations of cells					
FLC-5, M1, and A7	Human hepatoma cell line with murine cell lines (endothelial and stellate)	Radial flow			Alb production FLC-5 decreased by co-culturing	
HepG2-GS-CYP3A4 and PCTL-MDR	Modified hepatoma cell line with rabbit renal cell line transduced with MDR	Compartment bioreactor				
HLSC, stellate cells	Human liver stem cells with human stellate cells	Perfusion bioreactor wiht porous scaffold			Pos Alb	

* although the ATCC datasheet states that HepG2 does not form tumours in SCID mice, several investigators use HepG2 cells to induce tumour formation in SCID mice {Sun, 2004 459 /id;Lu, 2003 460 /id}.

** Ureagenesis can be positive without a functional urea cycle.

Abbreviations: AFP, alpha fetoprotein; Alb, albumin; ApoA1, apolipoprotein A-1; Arg, arginase; ALF, acute liver failure; AMC-BAL, Academic Medical Center-BAL; CYP, cytochrome P450; ELAD extracorporeal liver assist device; elim, elimination; GS, glutamine synthetase; HE, hepatic encephalopathy;

HNF4, hepatocyte nuclear factor 4; hTERT, human telomere reverse transcriptase; neg, negative; IL-1, interleukine 1; OTC, ornithine transcarbamoylase; pos, positive; pRb, Retinoblastoma protein; prod, production; PH, primary hepatocyte; PXR, pregnane X receptor; revers imm PHH, reversibly immortalized primary human hepatocytes; SV40, simian virus 40.

			Two animal studies. The first study in pigs with total liver ischemia (n=8-9): positive effect on survival time. The second study in dogs with total liver ischemia (n=5-7): no effect on survival time		Enosawa et al. Cell Transplant. 2000 Enosawa et al. Cell Transplant. 2001 Enosawa et al. Transplant Proc. 2001 Enosawa et al. Cell Transplant. 2006
			One animal study (n=7-8) in dogs with total liver ischemia: pos effect on survival time and diazepam metabolism		Wang et al. Artif Organs. 2005
					Coward et al. Artif Organs. 2009
		Lactate production and glucose consumption			Nibourg et al. Liver Transplant. 2010
					Akiyama et al. Int J Mol Med. 2004
			One animal study (n=9) in rats with D-galactosamine intoxication. No positive effects on survival. Significant reduction IL-6 levels.		Shinoda et al. J Surg Res. 2007
No OTC expression					Saito et al. World J Gastroenterol. 2006
		Lactate production			Endo et al. Art Org. 2002 Takahashi et al. Cell Transplant. 2006
					Carraro et al. Tissue Eng Part C. 2011

Tones of grey indicate the potential for BAL application as indicated by in vitro functionality or efficacy in animal studies. White: no data available. Darkest tone grey: in vitro functionality < 5% of primary hepatocytes, or not effective in animal study. Lightest tone grey: inconclusive. Middle tone grey: in vitro functionality > 10% of primary hepatocytes, or proven efficacy in animal study. * Although the ATCC datasheet states that HepG2 does not form tumours in SCID mice, several investigators use HepG2 cells to induce tumour formation in SCID mice.[101, 102] ** Ureagenesis can be positive without a functional urea cycle. Abbreviations: AFP, alpha fetoprotein; Alb, albumin; ApoA1, apolipoprotein A-1; Arg, arginase; ALF, acute liver failure; AMC-BAL, Academic Medical Center-BAL; CYP, cytochrome P450; ELAD extracorporeal liver assist device; elim, elimination; GS, glutamine synthetase; HE, hepatic encephalopathy; HNF4, hepatocyte nuclear factor 4; hTERT, human telomere reverse transcriptase; neg, negative; IL-1, interleukine 1; OTC, ornithine transcarbamoylase; pos, positive; pRb, Retinoblastoma protein; prod, production; PH, primary hepatocyte; PXR, pregnane X receptor; revers imm PHH, reversibly immortalized primary human hepatocytes; SV40, simian virus 40.

Primary human hepatocytes (PHHs) are the ideal cell source for BAL application and there have been case reports and a phase I study describing their safe use in ALF patients.[16, 17] However, due to limited availability, high variability and complex logistics the chances of a PHH-BAL eventually reaching the clinic are negligible. Alternatively, BALs based on primary porcine hepatocytes (PPHs) have proven efficacious in animal models of ALF.[18, 19] However, the use of animal-derived cells is compromised by xenotransplantation-related risks. Therefore, BAL devices can only be clinically successful when based on a functional human biocomponent that is readily available in large quantities, *i.e.* that proliferates *in vitro*.

Some previously published reviews discuss the potential of proliferative human cell sources for BAL application.[20, 21] However, none of these reviews focus on those cell sources that have actually been applied in BAL devices. These include hepatoma derived cell lines, *in vitro* immortalized hepatocytes, and immature cells such as fetal liver cells or stem cells. In addition, all these cell sources can be genetically modified to increase their performance, and combinations of different cell sources can be used (Table 1).

Therefore, the purpose of this review is to compare all proliferative human cell sources that have been applied in BAL systems and evaluate their potential. To this end, we searched the MED-LINE database on key words related BAL systems and human cells, and reviewed the published literature until July 2011. In addition, we included unpublished data from our own experiments.

ASSESMENT AND COMPARISON OF CELLULAR FUNCTIONALITY AND BIOARTIFICIAL LIVER SYSTEMS

Comparing the functionality of human bio-components in BAL systems entails some difficulties, since the performance of such BAL systems does not only depend on the biocomponent, but also on the BAL configuration, culture and/or test conditions (medium flow rate, static or dynamic culture). Therefore, proper comparison of the bio-components in different BAL devices is only possible when a study has incorporated a positive control group, *i.e.* freshly isolated primary mammalian hepatocytes (PHs) cultured similarly in the same device. In the case that these data are not available, a functional comparison was made upon the basis of literature data, and we feel that conclusions based upon these data must be drawn with reserve.

HEPATOMA CELL LINES

Hepatoma cell lines are derived from hepatoblastomas or hepatocellular carcinomas. The most frequently used hepatoma cell line for BAL application is the HepG2 cell line.[22] Nyberg et al. compared BALs with HepG2 cells or primary rat hepatocytes (PRHs).[23] In this study the detoxification function of PRH-BALs clearly outperformed that of HepG2-BALs, as demonstrated by an over 233-fold higher oxidation rate and a 30-fold higher glucuronidation rate. Ammonia elimination is highly variable between different HepG2-BALs ranging from 64% the rate of elimination of PPH-BALs to ammonia production. [24-26] Carbohydrate metabolism of HepG2 cells deviates from that of PHHs. HepG2-BALs produce lactate and consume glucose, whereas PHH-BALs consume lactate and produce glucose.[23, 25-27] Consumption of glucose and the subsequent conversion into lactate despite sufficient oxygen supply, is a well-known feature of tumor cells, known as the Warburg effect and may affect BAL culture and even stimulate lactic acidosis in SLF patients.[28] On the other hand, the synthetic functions of HepG2-BALs are markedly high with low variability between different BAL devices. In Nyberg's study, the rate of albumin synthesis reached an exemplary 74% of the rate in PRH-BALs. Others have reported synthesis of fibrinogen, ApoA1 and alpha-fetoprotein (AFP) in their HepG2-BALs.[26, 27, 29] However, it should be noted that AFP is typically an immature hepatocyte marker, and therefore has limited value. Only one group evaluated a HepG2-BAL in an animal study. Acetaminophen-overdosed rabbits were treated with alginate-encapsulated HepG2-beads.[30] This treatment resulted in some hemodynamic improvement, but ammonia levels, bilirubin levels and prothrombin time did not improve, and survival studies were not included.

To increase the functionality of HepG2, Kelly et al. selected clones for strong contact inhibition (exit of the cell cycle short after reaching confluence), high albumin production, high AFP production, and the ability to grow in glucose-deprived medium, yielding cell line C3A.[31] In a study by Carraro et al. C3A-BALs demonstrated CYP1A2, CYP2B6, and CYP2E1 activity, but this study lacks comparison with PH-BALs.[32] Both ammonia elimination and production are reported in different C3A-BALs, possibly related to the differences in BAL systems and culture environment.[33-36] Urea production in C3A cells is – at least on monolayer and similarly to HepG2 cells – a result of Arginase 2 (Arg2) activity, and not of a functional urea cycle.[37] C3A-BALs are capable of gluconeogenesis, as reported by Ellis et al. However, in other studies C3A-BALs consumed glucose and also produced lactate.[35, 36, 38] Synthetic functions of C3A-BALs are comparable to PH-BALs, including the production of albumin.[32, 35, 36, 38-41] Despite the lack of convincing *in vitro* data, C3A-BALs have been tested in animals and particularly in patients. In 1991 Kelly et al. reported on the treatment of anhepatic dogs with a hollow fiber BAL loaded with C3A cells, named the extracorporeal liver assist device (ELAD).[31] Three dogs were treated with this BAL system and 3 control dogs received supportive care. One dog treated with the ELAD lived 12.5 hours, whereas all other 5 lived 3 to 5 hours. In addition, BAL treatment

did not improve blood ammonia levels. In an uncontrolled follow up study with 3 dogs with acetaminophen-induced ALF, two survived the experiment.[42, 43] These small and uncontrolled studies do not justify any conclusions on efficacy of therapy. Nevertheless, patient studies with the ELAD were initiated. After a clinical phase I study, efficacy of the ELAD was tested in a randomized controlled pilot study in 17 patients with potentially recoverable ALF and 7 listed for LT.[44] In both groups, no effect of on survival, HE grade and blood ammonia levels was observed. After these studies, the ELAD was modified (ultrafiltrate instead of blood perfusion through the ELAD, increased fiber permeability and cell mass) and proved safe in a new phase I clinical trial.[45] A phase II controlled clinical trial performed in China followed, of which no results have been published in peer-reviewed journals yet. Currently, a follow-up phase II clinical trial has started in the US.

Next to HepG2 and C3A, a few other hepatoma cell lines have been applied in BAL systems. Yamashita et al. compared the Huh7 cell line with the HepG2 cell line and PPHs, cultured in a polyurethane foam/spheroid BAL system.[24] In this study, ammonia elimination and albumin production in the Huh7-BAL were 38% and 50%, respectively, of the PPH-BALs, but the HepG2-BALs outperformed the Huh7-BALs.

Three other hepatoma cell lines, named FLC-4, FLC-5, and FLC-7, were tested in radial flow BALs.[46] The expression levels of *albumin* and coagulation factors were <10% and 80% of the *in vivo* level, respectively. None of the cell lines expressed the complete set of genes involved in ammonium or drug metabolism. The FLC-4 radial flow bioreactor was further evaluated in a pig model of α-amanitin and lipopolysaccharide induced ALF. Both the two control pigs died in this study, whereas all three treated pigs survived.[47] Again however, the small numbers of experiments do not justify any conclusions on the efficacy of this FLC-4-BAL. The FLC-5-BAL and FLC-7-BALs were only tested in uncontrolled *in vitro* studies. The FLC-5-BAL expressed *CYP3A4* and showed functional CYP3A4 activity which increased substantially upon induction by rifampicin.[46] The FL-7-BAL produced albumin and AFP, and consumed glucose.[48] As mentioned above however, AFP production is a characteristic of fetal liver cells and not of mature hepatocytes, and glucose consumption (instead of production) does not add to its hepatospecificity.

Recently, the hepatoma cell line HepaRG has been evaluated for BAL application. The HepaRG cell line is a bipotent hepatocyte progenitor cell line that differentiates *in vitro* into two distinct types of cells: hepatocyte-like cells, and billiary-like cells upon reaching confluence in monolayer cultures, and further differentiation can be realized by culturing in the presence of 2% dimethyl sulfoxide (DMSO), a modulator of chromatin structure, for two weeks.[49] This way, the HepaRG cell line exhibits exceptionally high detoxification functionality *in vitro*, thereby providing a valuable tool for drug metabolism studies.[50] In a study by Darnell et al., HepaRG cells were cultured in a three-dimensional (3D) multi-compartment capillary membrane bioreactor.[51] The cells were proliferated for 2 weeks without DMSO, followed by a differentiation phase for

2 weeks in the presence of 2% DMSO. During the whole culture period, this HepaRG-BAL produced lactate and albumin. After differentiation, activities of CYP1A1/2, CYP2B6, CYP 2C9, and CYP3A4 were tested both 3-days and several weeks after omission of DMSO, and were found to be similar, except for CYP2B6, which increased. In this study, no PH-BAL was included and bioactive mass was not quantified, rendering quantitative interpretation of these results difficult. In our lab, we cultured the HepaRG cell line in the AMC-BAL according to a modified culture protocol without DMSO.[52, 53] After 14 culture days of BAL culture, CYP3A4 activity was 31% of the rate of PHHs cultures on monolayer. Ammonia elimination and ureagenesis were 144% and 23% of the rates of PPH-BALs, respectively. A ^{15}N ammonia loading experiment revealed that 27% of all formed urea was produced by a functioning urea cycle. In addition, the HepaRG-BAL demonstrated liver-specific carbohydrate metabolism by consumption of lactate (148% the rate of PPH-BALs). Finally, synthetic functionality of the HepaRG-BAL was also high as ApoA1 production reached 192% the rate of PHHs on monolayer. Subsequently, this HepaRG-BAL was tested in rats with ALF, induced by total liver ischemia (n=5-6). Treatment with HepaRG-BALs resulted in a significantly increased survival time of ~50%, and a delayed increase of HE, renal failure, and blood ammonia levels.

In conclusion, hepatoma cell lines show a large variety in their performance, particularly related to detoxification, nitrogen and carbohydrate metabolism. Of these cell lines the HepaRG cell line shows most promise, but still has to prove efficacy in a clinical trial. However, testing of the C3A cell line in the ELAD system has advanced most progressively by entering its third clinical phase II trial.

IN VITRO IMMORTALIZED CELLS

Immortalization implies overcoming normally occurring cell cycle arrests *in vitro*. Progression of the cell cycle from the G1 phase to the S phase is controlled by both positive (*e.g.* cyclins, cyclin-depent kinases, transcription factor E2) any negative regulators (*e.g.* tumor suppressor proteins p53, p21, p16, and the retinoblastoma protein (pRb)), and by telomere length [54, 55]. Strategies to immortalize human liver cells include manipulation of the expression of cell-cycle controlling genes and of *human telomerase reverse transcriptase (hTERT)*, the catalytic subunit of telomerase that restores telomere length after each cell division.

In a study from Werner et al., immortalized PHHs were generated by downregulating p53 and pRb, and by overexpressing the *EF2* and *cyclin D1* genes. This new cell line HepZ, was cultured in a bioreactor composed of microporous gelatin microcarriers.[56, 57] The HepZ-BAL consumed glutamine and glucose, and produced ammonia during the 10 days of culture. Lactate production was seen the first 7 days, but switched to a minor consumption during the last 3 days of culture. In addition, the HepZ-BAL displayed CYP activity by metabolizing lidocaine.

Unfortunately, this study was not PH-BAL controlled. Nonetheless, ammonia production obviously is an undesirable feature for a BAL.

In our lab, we generated the cBAL111 cell line by overexpressing *hTERT* in human fetal liver cells (FLCs).[58] cBAL111 cells were tested in the AMC-BAL at two different loading densities.[59] The high density cBAL111-BALs eliminated ammonia up to a level of 49% of PPH-BALs. As urea production only reached ~1% of the level of PPH-BALs and glutamine concentrations increased after ammonia loading, ammonia elimination was probably predominantly the result of GS activity. Albumin synthesis and detoxification function, as tested by lidocaine elimination, were low in cBAL111-BALs, with rates of 6% and 0.1% relative to PPH-BALs respectively. Furthermore, cBAL111-BALs were unable to prolong survival in a rat model of ALF (data not published).

Akiyama et al. evaluated the OUMS-29 cells and OUMS-29/H11 cells in a radial flow bioreactor.[60] The OUMS-29 cell line was generated by transfecting FLCs with the gene encoding the simian virus 40 large T antigen which perturbs p53 and pRb function.[61] The OUMS-29-BALs produced albumin at a rate of ~10% the rate of PRH-BALs in Nyberg's study.[23] In addition, no CYP3A4 protein was detected in OUMS-29-BALs.

Kobayashi et al. reported on the efficacy of human hepatocytes reversibly immortalized by overexpressing the *hTERT* under cre/loxP control.[62, 63] This elegant model allows expansion of the cells until sufficient bioactive mass, followed by inhibition of expansion by deletion of the *hTERT* gene through cre-recombinase activity, with concomitant differentiation and reduction of potential tumorigenesis. In a monkey model of *D*-galactosamine-induced ALF, empty BAL treated monkeys died within 5 days, whereas monkeys treated with BALs loaded with immortalized hepatocytes or PPHs (positive control) survived for over 14 days.[62] Unfortunately however, the number of animals used and the statistical analysis are lacking in this study. In a follow-up study Kobayashi et al. reported a significant improvement of survival and biochemical parameters in pigs with ALF when treated with a BAL loaded with reversibly immortalized hepatocytes.[63] Again however, detailed outcomes and statistical analysis were not provided.

In conclusion, the immortalized cell lines that have been applied in BALs so far display insufficient hepatic functionality, or lack necessary data on efficacy in animal models of SLF.

FETAL LIVER CELLS AND STEM CELLS

A third group of BAL biocomponents are immature human cells, including FLCs and stem cells from embryonic, mesenchymal and hepatic origin.

FLCs, in general harvested by digestion of fetal livers with collagenase followed by low-speed centrifugation, display sufficient proliferative capacity ***in vitro***, as they can undergo 58 population doublings before entering growth arrest.[64] We compared FLC-based AMC-BALs

cultured for 7 days with PHH-BALs and PPH-BALs.[65] Although both FLC-BALs and PPH-BALs produced the same amount of urea, FLC-BALs produced ammonia, in contrast to PPH-BALs. Albumin production was comparable in all three groups, and lidocaine elimination was over highest in FLC-BALs. Furthermore, FLC-BALs produced lactate, and switched to glucose consumption after 3 days of culture, probably reflecting dedifferentiation. Two other studies also reported lactate production in FLC-BALs.[66, 67] In addition, these studies reported albumin synthesis and *CYP3A4* and *CYP3A7* expression, although no quantitative comparison was made with mature hepatocytes.[66, 67]

Small hepatocytes (SHs), originally first identified in rats, are committed hepatocyte progenitor cells that can proliferate *in vitro* for over 3 months, and have the capacity to differentiate into mature hepatocytes *in vitro* [68]. In an uncontrolled study by Pavlic *et al.*, SHs were tested in a BAL prototype comprising a rotary cell culture system and displayed detoxification function by both eliminating diazepam and oxazepam, and metabolizing ethanol.[69] In addition, SH-BALs produced urea at approximately the same rate as the PRH-BALs in Nyberg's study, and albumin production reached twice the PRH-BAL rate.[23] Two follow-up studies by Wurm *et al.* confirmed the previously observed functionality.[70, 71] In one of these studies, ureagenesis could even be increased 3-fold upon a challenge with 1 mM ammonia. On the other hand, lactate was produced and morphine was not metabolized.[70, 71]

Another study by Fonsato *et al.* exploited human liver stem cells (HLSCs) that initially express mesenchymal and embryonic stem cell markers, and no oval cell markers.[72] BAL culture enhanced the differentiation of HLSCs substantially. They produced high amounts of hepatocyte growth factor (HGF), lost expression of stem cell markers and displayed CYP activity a rate comparable to PHH-BALs as observed by metabolism of ethoxy-4-trifluoromethylcoumarin. Furthermore, HLSC-BALs produced urea and albumin up to a rate of 51% and 69% of PHH-BALs, respectively. Notably, mesenchymal stem cells (MSCs) (see below) were cultured in the same BAL and MSC-BALs produced urea and albumin at a rate of 15% and 17%, respectively, of the PHH-BALs, which demonstrates that MSCs have less potential than HLSCs to differentiate into hepatocyte-like cells under this protocol.

Next to functional hepatic replacement, controlling inflammation can provide additive therapeutic support in SLF treatment. MSCs exert an immunomodulatory effect by inhibiting the function of various immune cells *in vitro.*[73] In a study by Parekkadan et al., MSC-BALs significantly increased the survival ratio (71% *versus* 14%) of rats with *D*-galactosamine-induced ALF (n=7) compared to fibroblast-BALs and empty BALs.[74] As treatment with MSC-conditioned medium resulted in significant improvement of survival as well, the effect is probably chemokine-related. In a study from Kazemnejad et al., human bone marrow-derived MSCs were cultured in a three-dimensional nanofibrous scaffold bioreactor and differentiated into hepatocyte-like cells using HGF, dexamethasone, and the cytokine oncostatin M.[75] In contrast to undifferentiated MSCs, MSC-bioreactors produced urea, aspartate aminotransferase,

alanine aminotransferase, and transferrin. Unfortunately, no PHH-BAL group was included as positive control, and concentrations of the abovementioned products were presented without a given volume, rendering quantitative comparison with PH-BALs impossible.

Recently, Miki et al. reported on human embryonic stem cells (hESC) that were cultured in a hollow fiber-based 3D perfusion bioreactor and differentiated into hepatocytes using a growth factor cocktail protocol.[76] They demonstrated that the induced 3D culture outperformed the 2D and uninduced controls in albumin and urea production, as well as the expression of several hepatic genes. However, the rate of albumin production in the induced hESC-BAL was less than 1% of the rate of the PRH-BAL in Nyberg's study.[23]

In conclusion, several types of immature cells offer interesting possibilities for BAL application. SHs and HLSCs both exhibit high hepatic functionality *in vitro*, although efficacy in animal models needs to be proven. MSCs have shown efficacy in an inflammatory model of ALF and might offer an interesting cell source for hepatic differentiation. FLCs are no likely candidates for BAL application, as they produce ammonia and dedifferentiate after a few days of culture.

FUNCTIONALY MODIFIED CELLS

The functionality of a potential BAL biocomponent can be increased by overexpressing structural hepatic genes or hepatic transcription factors. In addition, genes can be introduced with supportive characteristics, *e.g.* with anti-inflammatory or anti-apoptotic effects. An additional advantage of overexpressing a single gene, is that it allows an assessment of contribution of that particular function in BAL therapy by comparing the therapeutic effects of modified cell-BALs and their parental cell-BALs.

Modified hepatoma cell lines

As described above, HepG2-BALs display a low and inconsistent ammonia elimination, varying from ammonia production to reasonably high ammonia elimination. However, in most studies ammonia elimination and urea cycle activity are low. Therefore, Enosawa et al. overexpressed the *glutamine synthetase (GS)* gene, yielding the cell line HepG2-GS.[77] Although ammonia elimination through ureagenesis is preferable, as indicated previously, *GS* overexpression may increase the ammonia elimination. The HepG2-GS cell line was cultured in a radial flow bioreactor and the HepG2-GS-BALs eliminated ammonia at 15% the rate of PPH-BALs.[78] Next, this HepG2-GS-BAL was evaluated in ALF pigs with liver ischemia (n=8-9).[78-80] Mean survival was significantly higher in the HepG2-GS-BAL group compared to the empty BAL treated group.[78]

The same group further aimed to improve the HepG2-GS cell line by additionally overexpressing the *CYP3A4* gene, yielding the HepG2-GS-CYP3A4 cell line.[81] The HepG2-

GS-CYP3A4-BALs eliminated ammonia at a rate of 36% of PPH-BALs.[78] Next, treatment of ALF-dogs (induced by total liver ischemia and an additional overdose of diazepam) with a HepG2-GS-CYP3A4-BAL (n=8) resulted in a significantly longer surival time (42.7 hours) than treatment with a HepG2-GS-BAL (22.1 hours; n=7) or an empty BAL (31 hours; n=5). In addition, significantly more diazepam was metabolized in the HepG2-GS-CYP3A4-BAL group compared to both the control groups. These results underline the importance of detoxification function for a BAL. Interestingly however, this study reported no increased survival time by HepG2-GS-BAL treatment in contrast to a previous report.[78] Possibly this inconsistency is related to differences in the ALF models used. Nonetheless, further evaluation of the HepG2-GS cells and the HepG2-GS-CYP3A4 cells is necessary to investigate their efficacy, and the requirement of GS and CYP3A4 activity for BAL application.

By stably overexpressing the *pregnane X receptor (PXR)*, a key regulator of drug metabolism, in HepG2 cells, we undertook a different approach to overcome the low detoxification function of HepG2 cells.[26] In this new cell line, cBAL119, mRNA levels of CYP3A4, CYP3A5, and CYP3A7 increased 7- to 42-fold and CYP3A4 activity as measured by 6β-hydroxylation of testosterone was increased 4-fold *versus* HepG2. Unfortunately, CYP3A4 activity still reached only about 1 percent of that of PPHs cultured on monolayer. In addition, cBAL119 BALs did not eliminate ammonia nor produced urea. Therefore, cBAL119 most likely displays insufficient hepatic functionality for BAL application.

To increase ammonia elimination and urea production in HepG2 cells, Coward et al., overexpressed the genes encoding the urea cycle enzymes ornithine transcarbamylase (OTC) and arginase 1 (both limiting urea cycle activity in HepG2 cells), yielding the cell line HepG2-DT.[27, 37] In the HepG2-DT-BAL, incorporation of ^{15}N labeled ammonia into urea increased 10-fold, reaching 20% the level of PH monolayer cultures. The HepG2-DT-BALs produced the same amount of albumin as HepG2-BALs in this study. Although these results are promising, further functional improvements (detoxification, carbohydrate metabolism) are probably necessary to make HepG2 cells suitable for BAL application.

Modified *in vitro* immortalized cells

Shinoda et al. overexpressed the *interleukin 1 receptor antagonist (IL-1Ra)* gene in the conditionally immortalized cell line TTNT.[82, 83] IL-1Ra is a competitive inhibitor of IL-1, a very potent pro-inflammatory cytokine. IL1-Ra producing TTNT cells were cultured in a flat-plate BAL.[83] Treatment of *D*-galactosamine-induced ALF rats (n=9-14) did not significantly reduce hepatocellular leakage, nor improve the survival ratio at 28 days after treatment. In contrast, both treatment with a PRH-BAL overexpressing *IL-1Ra* as well as venous infusion of the IL-1Ra protein did increase the survival ratio in ALF rats.

The OUMS-29 cell line (described above) displayed low expression levels of *hepatic nuclear factor (HNF) 4A*, a major regulator of hepatic differentiation and no *CYP3A4* expression.[61]

Therefore, the OUMS-29/H-11 cell line was generated by overexpressing HNF4α.[60,84] In contrast to OUMS29-BALs, CYP3A4 protein was produced by OUMS-29/H-11-BALs; however, the authors state that the level was "much lower" than in human liver. OUMS-29/HNF-11-BALs also demonstrated 6β-hydroxylation of testosterone as a marker for CYP3A4 function in a late phase of the BAL culture; however, the activity was not quantified. OUMS-29/H-11-BALs have not been tested for efficacy in an animal model of SLF.

In conclusion, clear effects of overexpression of genes on BAL biocomponents are reported, and may certainly add to their functionality, if basic hepatic activity is present.

COMBINATIONS OF CELLS

Heterotypic interactions between hepatocytes and non-parenchymal liver cells are known to modulate cell growth and increase or stabilize hepatic functionality.[85] Therefore attempts have been made to co-culture hepatocytes in a BAL with supporting cells, like MSCs, hepatic stellate cells/cell lines, or endothelial cells/cell lines. Some of these BALs will probably not reach clinical application, as they currently rely on animal cells and/or PHs. Nevertheless, we will discuss four examples to illustrate their potential.

In a study by Saito et al., the hepatoma cell line FLC-5 was co-cultured in a radial flow bioreactor with the mouse endothelial cell line M1 and the mouse hepatic stellate cell line A7.[86] Compared to a FLC-5-BAL, expression levels of *albumin* and *HNF4A* decreased significantly in the FLC-5/M1/A7-BAL, suggesting a dedifferentiation and/or suppressing effect of M1/A7 co-culture. Although the FLC-5/M1/A7-BAL, in contrast to the FLC-5-BAL, produced urea, gene expression analysis of the urea cycle genes revealed no expression of *OTC*. Therefore the observed urea production is not the result of urea cycle activity, but most likely of non-hepatocyte specific arginase 2 activity.

Endo et al. evaluated the functionality of a double-compartment BAL in which the PCTL-MDR cell line, a rabbit proximal tubule (kidney) cell line overexpressing the apical efflux transporter multidrug resistance protein 1 (MDR1), was co-cultured with the HepG2 or the HepG2-CYP3A4 cell lines.[87] The rationale for the use of the PCTL-MDR cells was that these cells are capable of transporting the drugs digoxin and doxorubicin solely in a basolateral-to-apical direction.[88] The PCTL-MDR cells were cultured on a tight monolayer on one side of the double-compartment BAL, and HepG2-GS or HepG2-GS-CYP3A4 cells were cultured on the opposite side. Although they do not present data, the authors state that testosterone hydroxylation was "very low" in het PCTL-MDR/HepG2-GS-BAL, whereas it was "almost equal to isolated human hepatocytes" in the PCTL-MDR/HepG2-GS-CYP3A4-BAL. Another study with the same PCTL-MDR/HepG2-GS-CYP3A4-BAL reported lidocaine metabolism and selective transport from the outer to the inner compartment (not quantified).[89] In this study,

the lactate concentration increased on the PCTL-MDR side, whereas the concentration on the HepG2-GS-CYP3A4 side remained constant. The ammonia concentration decreased on the HepG2-GS-CYP3A4 side and remained constant on the PCRL-MDR side. Nonetheless, the ammonia elimination rate only reached ~1% of PPHs on monolayer.[90]

In a study by Yagi et al., PRHs were co-cultured with MSCs to enhance hepatic functionality and introduce anti-inflammatory activity.[91] On monolayer PRH-MSCs co-cultures showed increased albumin secretion and CYP1A1 activity compared to PRH cultures. In addition, treatment of *D*-galactosamine-induced ALF rats with a PRH-MSC-BAL (n=9) increased their 20-day survival ratio substantially compared to fibroblast/PRH BAL treatment (n=10). Separate MSC-BAL and PPH-BAL groups were not included in this study, which would have been interesting to isolate the effect of co-culture. Nonetheless, this study underlines the potential of MSCs to stabilize and increase hepatic functionality of the co-cultured cells.

In a recent study by Carraro et al., HLSCs were co-cultured with hepatic stellate cells in a perfused bioreactor.[92] The co-culture secreted albumin at a maximally 1.4-fold increased rate. No other liver-specific functions were assessed.

In conclusion, co-culturing can negatively and positively affect the functionality of hepatocytes and add useful non-hepatic functions, such as anti-inflammatory activity. In this respect, co-cultures of hepatocyte-like cells with MSCs as supportive biocomponent are a promising option.

CONCLUSION

Proliferative human cell sources offer a promising alternative to PHs for BAL application. The hepatoma cell line HepaRG, the modified hepatoma cell line HepG2-GS-CYP3A4, and the differentiated immature cells SHs and HLSCs all demonstrate a substantial level of *in vitro* hepatic functionality when compared to PHs, the golden standard. In addition, HepaRG-BALs, HepG2-GS-CYP3A4-BALs, and MSC-BALs have proven efficacy in well-powered animal studies of ALF. Co-culturing of hepatocytes with supportive cells can improve hepatic functionality and add useful other functions such as anti-inflammatory characteristics. Lastly, only one non-primary human cell-based BAL (the ELAD) has progressed to a clinical trial so far that still awaits its results to be published.

EXPERT OPINION

All of the discussed hepatocyte-like biocomponents show deficiencies in their hepatic functionality compared to PHs. This is probably (at least partly) related to their proliferative capacity and the mutual exclusivity of proliferation and differentiation as found in hepatocytes and other cell sources.[93-96] A combination of paracrine and autocrine factors and signals from cell-cell and cell-extracellular matrix interactions, *e.g.* E-cadherin, direct several intracellular signaling pathways, including the Wnt pathway, eventually determine this balance. To circumvent the consequences of the reciprocal relationship between proliferation and differentiation, a number of strategies can be been applied.

By overloading the BAL with cells, differentiation can be promoted and proliferation inhibited, as high cell density promotes the establishment of cell-cell contacts, which induces hepatic differentiation.[93] So far, the relation between cell density and differentiation of proliferative biocomponents in BAL devices has not been studied extensively. To our knowledge, our study with two different cBAL111 cell-masses cultured in the AMC-BAL was the only one, indeed showing increased functionality in the high density cBAL111-BAL *versus* the low density cBAL111-BAL.[59]

Furthermore, hepatic differentiation can be induced by differentiation-inducing growth factors such as HGF, epidermal growth factor, fibroblast growth factor-4, insulin, corticosteroids, and modulators of chromatin structure such as DMSO and butyrate.[49, 94, 95] So far, these compounds have seldom been included in the medium for BAL cultures. Another option is to develop a reversibly immortalized cell line. Kobayashi et al. developed such a reversibly immortalization cell line using the *hTERT* gene under cre-loxP control.[62, 63] Unfortunately, detailed information on the functionality of this cell line is still lacking.

Hepatic differentiation can also be increased by co-culturing hepatocyte-like cells with supporting cells. Promising results have been reported in this respect by coculturing PRHs with MSCs that not only added anti-inflammatory characteristics but also increased albumin secretion.[91] However, coculturing in a BAL system is complex and may even yield negative results as illustrated by the study on the FLC-5/M1/A7-BAL.[86]

Finally, genetic modification may increase hepatic functionality, as illustrated by the beneficial effects of overexpression of *OTC*, *ARG1* and *PXR* in HepG2 cells, and of *HNF4A* in OUMS-29 cells.[26, 27, 60] Moreover, BALs based on HepG2 cells overexpressing *GS* and *CYP3A4* have (in contrast to a HepGs-BAL) proven efficacies in an animal model of ALF, underscoring the importance of ammonia elimination and CYP mediated detoxification in BAL therapy.[78, 81]

All these methods to improve hepatic functionality will, however, only be effective when the biocomponent already displays a basic level of hepatic differentiation. With our current knowledge on hepatic differentiation we are still unable to effectively improve biocomponents with low hepatic functionality. Our study on a previously developed, reversibly immortalized cell

line, NKNT-3, on monolayer is exemplary.[34, 97] Reversion of immortalization increased mRNA levels of albumin, transferrin and α-1-antitrypsin 4- to 20-fold, however, these levels reached maximally 0.1% the level of human liver cells.

Hypothetically, different cell sources may be combined in one BAL to yield a more all-round hepatic functionality. However, from Table 1 it is clear that most biocomponents do not exhibit complementary phenotypes. Instead, most cell sources display synthetic functionality, as determined by *albumin* expression, and on the other hand share deficiencies in CYP activity, nitrogen and carbohydrate metabolism. This phenomenon probably relates to the liver development *in vivo*, during which *albumin* is already expressed early whereas, for instance, *CYP* genes and the genes encoding the urea cycle enzyme CPS are only expressed late during differentiation.[95, 98]

An important issue regarding BAL therapy is safety. Every cell with unlimited proliferating potential *in vitro* (particularly cell lines and undifferentiated stem cells) has tumorigenic potential when escaping into the patient's body during treatment. Therefore, the incorporation of barriers between the cells and the patients is of paramount importance. Genetic modification provides an additional tool to diminish the risk of tumorigenesis by *e.g.* expression of a suicide gene, such as the *herpes simplex virus type 1 thymidine kinase (HSV-tk)* gene, that increases the cytotoxicity of the antiviral ganciclovir, enabling efficient elimination of these cells when necessary, as shown in OUMS-29 monolayer cultures. [99]

Taken together, our findings show that several proliferative human cell sources offer a promising alternative to PH cells for BAL application. As yet however, no BAL has proven efficacy in a large randomized controlled clinical trial. Therefore, clinical implementation of a proliferative human cell-based BAL is at least still several years away. Nonetheless, we are confident that the major unmet clinical need to expand our repertoire of therapies for SLF patients will be the driving force to further improve and clinically test the recently developed, promising BALs based on proliferative human biocomponents.

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CHAPTER 3

Stable overexpression of *pregnane X receptor* in HepG2 cells increases its potential for bioartificial liver application

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ABSTRACT

Background: To bridge acute liver failure patients to transplantation or liver regeneration a bioartificial liver (BAL) is urgently needed. Such a BAL consists of an extracorporeal bioreactor loaded with a bioactive mass that should preferably be of human origin and display high hepatic functionality, including detoxification. The human hepatoma cell line HepG2 exhibits many hepatic functions, but its detoxification function is low.

Aim: In this study, we investigated whether stable overexpression of *pregnane X receptor (PXR)*, as a master regulator of diverse detoxification functions in the liver, *e.g.* cytochrome P450 3A (CYP3A) activity, increases the potential of HepG2 for BAL application.

Methods & Results: Stable overexpression was achieved by lentiviral expression of the human *PXR* gene, yielding cell line cBAL119. In monolayer cultures of cBAL119 cells, *PXR* transcript levels increased 29-fold compared to HepG2 cells. Upon activation of *PXR* by rifampicine, the mRNA levels of CYP3A4, CYP3A5 and CYP3A7 increased 49- to 213-fold compared to HepG2 cells. Using reporter gene assays with different inducers, the highest increase in *CYP3A4* promoter activity (131-fold) was observed upon induction with rifampicin. Inside the BAL, the proliferation rate as measured by DNA content was comparable between the two cell lines. The rate of testosterone 6β-hydroxylation, as a measure for CYP3A function inside the BAL, was 4-fold increased in cBAL119-AMC-BALs compared to HepG2-AMC-BALs. Other functions, like apolipoprotein A-1 synthesis, urea synthesis, glucose consumption and lactate production remained unchanged or increased.

Conclusions: Thus, stable *PXR* overexpression markedly increases the potential of HepG2 for BAL application.

INTRODUCTION

Acute liver failure (ALF) is a severe syndrome with mortality rates as high as 80%. Despite the progress made in supportive care, liver transplantation is the only curative treatment option to date. Liver transplantation is, however, restricted by the limited number of donor organs.[1] To bridge these patients to transplantation, bioartificial liver (BAL) support systems have been developed.[2] BAL systems comprise an extracorporeal bioreactor loaded with hepatocytes that is connected to the patient's circulation. The functionality of these systems obviously relies on the hepatic functionality of the applied cells.

One of the developed BAL systems is the AMC-BAL which showed efficacy in several animal models of ALF and safety in a clinical phase I trial.[3] This device and most other BAL systems rely on primary porcine hepatocytes, but the clinical application of xenogenic cells is hampered by xenotransplantation related risks.[4, 5] A cell source of human origin should therefore be more suitable for BAL application. A human cell source should exhibit both sufficient *in vitro* hepatic functionality and proliferative capacity. Human liver cell lines are therefore a promising cell source for BAL use.

HepG2 is a human hepatoma cell line, derived from a well-differentiated hepatocellular carcinoma of a 15-year-old Caucasian male.[6] This cell line synthesizes a variety of hepatic proteins, like albumin and transferring.[7] However, HepG2 has a marginal detoxification function, as it lacks functional expression of almost all the relevant *cytochrome P450 (CYP)* genes.[8] In ALF patients though, a myriad of toxic compounds accumulate in the circulation, and therefore a properly functioning detoxification system is a prerequisite for a cell source to be used in a BAL. Accordingly, in a dog model of ALF, treatment with a bioreactor loaded with HepG2 cells overexpressing CYP3A4, resulted in increased survival in comparison with dogs treated with a bioreactor containing the parental HepG2 cells [9].

Hepatic detoxification encompasses the biotransformation of hydrophobic toxins into water soluble substances, which can be secreted into urine or bile. It comprises four phases: hepatic uptake, intracellular modification and subsequent conjugation, and finally secretion. Intracellular modification is typically a hydroxylation reaction carried out by the large family of CYPs. The CYP3A family is the most dominant one on both the level of expression and potency, as it metabolizes approximately 50% of all xenobiotics.[10]

Regulation of hepatic detoxification at the molecular level involves a complex interplay between toxic agents (endo- and xenobiotics), transcription factors, including nuclear receptors and coreceptors, metabolizing and conjugating enzymes, and import and export transporters. Nuclear receptors, crucial for the regulation of the hepatic detoxification, include the pregnane X receptor (PXR, NR1I2), Hepatocyte nuclear factor 4 alpha (HNF4A) and constitutive androstane receptor (CAR, NR1I3).[11, 12] PXR regulates hepatic detoxification at every level. It controls the expression of genes encoding various transporters (both import and export),

CYP genes including the *CYP3A* family, and genes encoding conjugation enzymes.[13] To exert its effects, PXR first needs to bind to an activating ligand, such as rifampicin or dexamethasone. Subsequently, this complex translocates to the nucleus, where it heterodimerizes with the retinoic X receptor, and then interacts with the regulatory sequences of its target genes.[11]

Rifampicin is a very potent activator of PXR in both human hepatocytes and HepG2 cells. Using transient transfection assays, several studies showed an increase in CYP3A4 promoter activity in HepG2 cells upon addition of rifampicin. However, the extent of CYP3A4 promoter activation in these studies varied from 5-fold up to 20-fold. Possibly, this is due to differences in cell culture conditions or the used HepG2 cell line.[14-16]

In stably transfected HepG2 cells overexpressing PXR (DPX-2 cell line), rifampicin increased CYP3A4 promoter activity 35-fold and CYP3A4 metabolic activity 3- to 5-fold compared to non-induced cells.[16, 17] However, both these studies lack the comparison with the ancestral HepG2 cell line, and thus the effect of *PXR* overexpression on the detoxification function remains unknown. In addition, other hepatic functions and the proliferative capacity of the cells were not studied in these studies. Consequently, the previous results do not unambiguously indicate the potency of *PXR* overexpression in HepG2 cells for BAL application.

Therefore, we stably overexpressed the human *PXR* gene in HepG2 cells by lentiviral transduction, yielding the new cell line cBAL119. We compared the functionality of cBAL119 with the parental HepG2 cells, and studied its potential for BAL application by characterizing its hepatic functionality, metabolism and proliferative capacity, both in monolayer as well as in laboratory models of the AMC-BAL.[18]

METHODS

Generation of cBAL119

The cDNA of the human PXR gene, kindly provided by Dr. Jean Marc Pascussie, (University of Montpellier, France) was introduced in HepG2 cells by lentiviral transduction. The lentiviral vector backbone was described as prrlcpptpgkgfppressin by Van Til *et al.*[19] A *puromycin resistance* gene preceded by a phosphoglycerate kinase (PGK) promoter was inserted into the *Eco* RV site and the *green fluorescent protein* gene was replaced by the human *PXR* gene. This way, a lentiviral vector, pBAL119, was generated containing two PGK promoters controlling the expression of both the *PXR* and *puromycin resistance* gene. The construct was confirmed by DNA sequence analysis.

The lentivirus was produced essentially as previously described.[20] In brief, HEK293T cells were transiently transfected using SuperFect Transfection Reagent (Qiagen) with a third generation lentiviral vector system, including pBAL119. Virus containing supernatant was collected at 24, 48 and 72 hours following transfection, filtered through 0.45 μm Millipore

filters and immediately transferred to HepG2 cells in subconfluent state in 6-well plates in a 1:1 (lentivirus preparation:culture medium) ratio. The cells were cultured in DMEM culture medium (Dulbecco's Modified Eagle's Medium with 10% heat inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U $\cdot mL^{-1}$ penicillin and 100 µg $\cdot mL^{-1}$ streptomycin (penicillin/streptomycin mix) (all reagents from Lonza)). Three days after transduction, the cells were passaged to a T75 flask and 20 µg $\cdot mL^{-1}$ puromycin was included in the culture medium to select for HepG2 cells with integrated lentiviral construct, this way yielding the polyclonal cell line cBAL119.

Monolayer culture and cell isolation

The cBAL119 and parental HepG2 cells were cultured in DMEM culture medium at 37°C in a humidified atmosphere (95% air, 5% CO_2). For large-scale expansion, the cell lines were cultured in CellSTACK® culture chambers (5-stacked; Corning). Culture medium was refreshed every three days. Cells were detached with a mixture of 50% 0.25% trypsin / 0.03% EDTA (Lonza) and 50% Accumax (Innovative Cell Technologies) (v/v), centrifuged at 50x g for 5 min and washed 2x with ice-cold DMEM culture medium and once in cold WE culture medium (Williams' E medium (Lonza) supplemented with 4% (v/v) heat inactivated FBS, 2 mM glutamine, 20 mU $\cdot mL^{-1}$ insulin (Novo Nordisk), 2 mM ornithine hydrochloride (Sigma-Aldrich), and 100 U $\cdot mL^{-1}$ penicillin and 100 µg $\cdot mL^{-1}$ streptomycin (penicillin/streptomycin mix)).

Reverse transcription PCR (RT-PCR)

RNA was isolated from the cBAL119 and parental HepG2 cells cultured to confluence in monolayer (6-well plates) by using the RNeasy mini kit (Qiagen). As a reference, two different human liver samples were included in the analyses. First strand cDNA was generated from 1 µg of total RNA using a RT primer mix, comprising 11 pmol of the 18S ribosomal RNA antisense primer and 25 pmol of gene-specific RT primers targeting to genes indicated in Table 1, with 134 units Superscript III (Invitrogen) in a total volume of 25 µL at 50 ºC for one hour. The reaction mix was heated to 70 ºC for 15 min and then cooled to 4 ºC. The resulting gene-specific RT reaction mixes were column purified to remove gene-specific primers using Qiaquick PCR Purification Kit (Qiagen) and were brought to a volume of 100 µL.

Real-time PCR using LightCycler®480 SYBR Green kit (Roche) was performed on 1 µL of RT-reaction mix according to manufacturers' instructions. Primers sequences and amplicon sizes are depicted in Table 1. The thermal cycling profile of the touchdown PCR was as follows: denaturation at 95 °C for 3 min, followed by 41 cycles of 94 °C for 1 s, primer annealing at 68°C for 7 s with a 0.5 °C $\cdot cycle^{-1}$ decrease until 63°C, and extension for 20 s at 72 °C.

Transcript levels were calculated as described, subsequently normalized for 18S ribosomal RNA and expressed as a percentage of the mean starting levels of two human liver samples.[21]

Table 1. Primer sequences used in the RT-PCR analyses with resulting amplicon sizes. Primers are indicated from 5' → 3'.

Gene	RT primer	Sense primer	Antisense primer	Amplicon size (bp)
18S rRNA	CGAACCTCCGACTTTCGTTT	TTCGGAACTGAGGCCATGAT	CGAACCTCCGACTTTCGTTCT	151
PXR	TCTGGTCCTCGATGGGCAAGTC	GAGAGCGGCATGAAGAAGGAGA	CATGTGGGGCAGCAGGGAGAAG	420
HNF4A	CACTCCAACCCCGCCCCTC	CCGGGTGTCCATACGCATCCT	CAGGTTGTCAATCTTGGCC	321
CAR	GCTCTTCTTGCTCCTTACTC	CGTCATGGCCAGTAGGGAAG	CATGCCAGCATCTAAGCACT	232
CYP3A4	CCCGTGAGAAGCAGAGGA	AGCTTAGGAGGACTTCTTCAACC	AGCCAAATCTACCTCCTCACACT	313
CYP3A5	GGTACCATCTCTTGAATCCACC	TGACCCAAAGTACTGGACAG	TGAAGAAGTCCTTGCGTGTC	240
CYP3A7	AGCCAAATCTACTTCCCCAGCAC	ATTACGCTTTGGAGGACTTCTTCT	CGTCTTCATTTCAGGGTTCTATTT	182
ALB	ACTTCCAGAGCTGAAAAGCATGGTC	TGAGCAGCTTGGAGAGTACA	GTTCAGGACCACGGATAGAT	189
CPS	CAGCTGTCCTCCGAATCAC	GAAGGGGCCCGAGAAGTAGAA	CTCAACCGGGGCCAGGAAAAC	445
ARG1	TGTGATTACCCTCCCGAGCAAGTC	TTGGCAAGGTGATGGAAGAAACA	CCTCCCGAGCAAGTCCGAAACAA	305
GS	TTGGCAGAGGGGCGACGAT	GCCTGCTTGTATGCTGGAGTC	GGCGCTACGATTGGCTACAC	420

Abbreviations: bp, base pairs; rRNA, ribosomal RNA; *PXR, pregnane X receptor; HNF4A, hepatic nuclear factor 4 alpha; CAR, constitutive androstane receptor; CYP3A4, cytochrome p450 3A4; CYP3A5, cytochrome p450 3A5; CYP3A7, cytochrome p450 3A7; ALB, albumin; ARG1, arginase 1; CPS, carbamoyl phosphate synthetase; GS, glutamine synthetase.*

CYP3A4 promoter activity analysis

HepG2 and cBAL119 cells were seeded in 24-well plates at 45% confluence. After 2 days the cells were transfected with plasmids pRL-TK (0.27 μg·well^{-1}) and CYP3A4-XREM-Luc (5.5 μg·well^{-1}), which were kindly provided by Dr. Richard Kim (University of Western Ontario, London, Ontario, Canada) using Lipofectamine™ 2000 (Invitrogen).[15] After transfection, the cells were cultured in 500 μL WE culture medium including 1 μL of different concentrations of inducers, *i.e.* rifampicin (Sigma), dexamethasone (Centrafarm) and ursodeoxycholate (UDCA) or dimethyl sulfoxide (DMSO) as vehicle control (all n = 3). After 16 hours, the medium including additions was refreshed and 24 hours later the relative CYP3A4 promoter activity was assessed with the Dual Luciferase Reporter Assay Kit (Promega).

Bioreactor

We used the laboratory-scale AMC-BAL bioreactor, which is a 55x down-scaled bioreactor of the third generation AMC-BAL with an internal volume of 10 mL.[22] The general configuration of the bioreactor consists of a polycarbonate housing, containing a three-dimensional non-woven hydrophilic polyester matrix circularly wound around a polycarbonate core. Between the matrix layers, hydrophobic polypropylene gas capillaries are situated in a parallel fashion. The ends of these capillaries are embedded in polyurethane resin and fitted with gas inlet and outlet caps.[18]

Bioreactor culture

Bioreactors were loaded with 9 ml cell suspensions, containing 1.6 mL cell pellet of HepG2 or cBAL119 cells (both n = 6). Loaded bioreactors were placed in a temperature controlled culture cabinet (37°C) and oxygenated with a mixture of 40% O_2, 55% N_2 and 5% CO_2 (v/v). Bioreactors were subjected to a 340° transverse and longitudinal rotation for 3 hours to ensure optimal cell attachment and an even cell distribution. After this attachment period, dead and unattached cells were removed by a flush of 40 mL culture medium, after which the bioreactors were continuously perfused with 100 mL recirculating BAL culture medium (WE culture medium with 10% (v/v) FBS instead of 4%, 1 μM dexamethasone, and without phenol red) supplemented with 10 μM rifampicin at 5 mL·min^{-1}. Every day, culture medium was completely refreshed.

Determination of bioactive mass

To determine bioactive mass, we investigated samples of the cell suspensions loaded into the bioreactors for cell pellet volume after centrifugation, cell counts, aspartate aminotransferase (AST) content, lactate dehydrogenase (LDH) content, total protein content, and total DNA content (all n = 4 per cell line). Cell pellet volume was determined after centrifugation at 50 g for 3 min. Cell counts were performed using a Bürker Bright line cytometer (Optik Labor). Every

cell suspension sample was counted twice and means were used for calculations. For intracellular AST and LDH content, 100 μL cell suspension was lysed by adding 900 μL 2% Triton X (Sigma Aldrich) and subsequent vortexing. These lysates were diluted 100x and measured for AST and LDH content as described previously.[23] Total protein and DNA content were measured in cell pellets obtained from 100 μL cell suspensions by centrifugation at 50 g for 3 min. Cell pellets were washed in PBS and recentrifuged twice before pellets were incubated overnight in 1 mL 0.2 M NaOH (Merck) at 37 °C. In these lysates, the total protein content was measured using the Coomassie Brilliant Blue G-250 staining kit (Bio-Rad), and the total DNA content was measured using the Hoechst 33258 reagent as described by Downs *et al.*[24] To measure proliferation inside the BAL, total DNA content was also analyzed in HepG2-AMC-BALs and cBAL119-AMC-BALs (n = 5 both) after a 4-day culture period. Therefore, both ends of all bioreactors were removed after 4 days of culture, and subsequently the total BAL contents were removed from the housing and shaken in 35 mL of 0.2 M NaOH at 37° for 3 days. After 3 days the lysate was harvested and the extraction was repeated twice for 3 consecutive days with fresh NaOH. The DNA and protein content was determined in all three lysates and the total DNA and protein content / BAL was then calculated. The last lysate did not contain any detectable protein or DNA.

Bioreactor function tests

Function tests were performed with test medium composed of BAL culture medium supplemented with 125 μM testosterone (Fluka) or 100 μM 7-benzyloxy-4-trifluoromethylcoumarin (BFC), 2 mM *L*-lactate (Sigma), 2.75 mM *D*-galactose (Sigma Aldrich) and 5 mM NH_4Cl (Merck). Rifampicin was omitted from the test medium, as it disturbs the measurement of urea. Bioreactors were first flushed with 30 ml test medium, followed by a 3-hour period of recirculation with 41 ml of test medium. Samples (1 mL) were taken at 30, 60, 120 and 180 min of recirculation and analyzed for concentrations of urea, glucose, lactate, as well as activities of AST and LDH as previously described.[23, 25] 6β-Hydroxytestosterone production was measured by high performance liquid chromatography coupled to mass spectrometry. Conversion of BFC to 7-hydroxy-4-trifluoromethylcoumarin (HFC) was measured fluorometrically at excitation wavelength 410 nm and emission wavelength 510 nm, after deconjugation of the samples with β-glucuronidase/arylsulfatase (150 Fisherman / 1200 Roy units) (Roche) at 37 °C for 2 hours and termination of the reaction in 0,25M Tris / 60% (v/v) acetonitrile.[26] Apolipoprotein A-1 (ApoA-1) production was assessed by an enzyme-linked immunosorbant assay using a polyclonal rabbit anti-human ApoA1 (Calbiochem) in a 1:500 dilution as primary antibody, a monoclonal mouse anti-human ApoA1 (Calbiochem) in a 1:500 dilution as secondary antibody, and a polyclonal rabbit anti-mouse IgG horseradish peroxidase (Dako) in a 1:2000 dilution as tertiary antibody. The reaction was developed with 3,3'-diaminobenzidine as chromogen. Function parameter rates were determined by calculating the changes in concentration in medium per hour per g of protein loaded into the BALs.

Statistical analysis

Repeated measurement analysis of variance tests were used to compare differences between the two bioreactor groups (HepG2 and cBAL119) over the 4 day culture period. Paired Student's t-tests were performed to compare daily differences within one experimental group. Unpaired Student's t-tests were performed to compare differences between two experimental groups per day. Factor correction was used to correct for between-session variation in multi-session experiments.[27] 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

Transcript levels of *PXR* and target genes are increased in cBAL119 cells

A cell line, named cBAL119, was generated by transduction of HepG2 cells with a lentiviral construct carrying the human *PXR* gene as well as the puromycin resistance gene. Selection for puromycin resistance generated >50 colonies, together yielding cell line cBAL119.

Subsequently, transcription levels of various hepatic genes were determined in monolayer cultures of HepG2 and cBAL119 in the absence and presence of rifampicin (Table 2). Expression of *PXR* in uninduced cBAL119 cells was increased 29-fold compared to the parental HepG2 cells, and up to 583% of the *in vivo* level, indicating establishment of *PXR* overexpression. As expected, addition of rifampicin had no effect on transcript levels of *PXR* in both cell lines. The mRNA levels of two other relevant nuclear hormone receptors, *i.e. HNF4A* and *CAR*, were not changed upon overexpression or activation of *PXR*. The mRNA level of HNF4A was with 484% of the *in vivo* level already high in HepG2 cells, whereas the *CAR* transcript level was below 1% of the *in vivo* level.

Concerning the PXR target genes of the CYP3A family, we observed a significant 18- and 7-fold increase of CYP3A5 and CYP3A7 mRNA levels in cBAL119 cells respectively, to 23%-24% of *in vivo* levels. Upon addition of rifampicin, transcript levels of *CYP3A5* and *CYP3A7* further increased in cBAL119, to 64% and 766% of the *in vivo* levels. The transcript level of *CYP3A4* was significantly (42-fold) increased in cBAL119 *versus* HepG2 only after addition of rifampicin. However, the transcription level remained low in induced cBAL119 cells, with <1% of the *in vivo* level.

Table 2. Transcript levels of several hepatic genes in HepG2 cells and cBAL119 cells.

	PXR	*HNF4A*	*CAR*	*CYP3A4*	*CYP3A5*	*CYP3A7*	*ALB*	*CPS*	*ARG1*	*GS*
HepG2 w/o rif	20.2 ± 8.2	484.2 ± 181.3	0.5 ± 0.2	0.006 ± 0.004	1.3 ± 0.3	3.6 ± 3.1	10.5 ± 8.1	3.8 ± 1.1	11.2 ± 5.1	1564.2 ± 623.8
HepG2 w/ rif	28.6 ± 4.4	664.7 ± 198.1	0.6 ± 0.4	0.022 ± 0.010[a]	4.3 ± 2.3[a]	6.5 ± 2.6	17.8 ± 15.3	5.1 ± 1.1	9.7 ± 4.8[a]	919.8 ± 200.2
cBAL119 w/o rif	583.0 ± 363.3[a,b]	389.4 ± 70.3[b]	0.8 ± 0.4	0.012 ± 0.004	23.5 ± 6.7[a,b]	23.7 ± 6.2[a,b]	55.9 ± 22.9[a]	3.4 ± 1.6	11.2 ± 3.7	925.4 ± 580.4
cBAL119 w/ rif	535.4 ± 210.9[a,b]	419.8 ± 257.6	0.6 ± 0.4	0.926 ± 0.402[a,b,c]	64.3 ± 42.9[a,b]	766.3 ± 724.1	35.4 ± 23.9	1.6 ± 0.1[a,b]	11.7 ± 11.7	341.0 ± 79.9[a,b]

Abbreviations: *PXR, pregnane X receptor; HNF4A, hepatic nuclear factor 4 alpha; CAR, constitutive androstane receptor; CYP3A4, cytochrome p450 3A4; CYP3A5, cytochrome p450 3A5; CYP3A7, cytochrome p450 3A7; ALB, albumin; ARG1, arginase 1; CPS, carbamoyl phosphate synthetase; GS, glutamine synthetase*; rif, rifampicin. Data are obtained in 4 separate experiments and corrected for between-session variation as described. Values are expressed as % of human *in vivo* levels and as means ± standard deviations. Significance: [a]$p < 0.05$ denoted group *versus* HepG2 w/o rif, [b]$p < 0.05$ denoted group *versus* HepG2 w/ rif, [c]$p < 0.05$ denoted group *versus* cBAL119 w/o rif.

Differential effects on transcript levels of other hepatic genes in cBAL119 cells

The transcript levels of other hepatic genes considered to be relevant for BAL application were also investigated. These encoded albumin (ALB) as a synthetic marker, two urea cycle enzymes carbamoyl phosphate synthetase (CPS) and arginase 1 (ARG1), as well as glutamine synthetase (GS), which, in addition to the urea cycle, is relevant for the elimination of ammonia. Unexpectedly, the *ALB* transcript levels were increased 5-fold (significant) in uninduced cBAL119, to 55% of *in vivo* levels. The transcript levels of *CPS*, *ARG1* and *GS* were unchanged in cBAL119 *versus* HepG2. The mRNA levels of ARG1 and CPS were both low in uninduced cBAL119 cells, being 11.2% and 3.1% of *in vivo* levels respectively, whereas the *GS* transcript levels were 925%. The addition of rifampicin resulted in a trend to diminished transcript levels of *ALB*, *CPS* and *GS* in cBAL119.

***CYP3A4* promoter activity is increased in cBAL119 cells and rifampicin is the most potent inducer**

To test the induction capacity of different inducers, *CYP3A4* promoter activity was assessed in both HepG2 cells and cBAL119 cells using a luciferase reporter gene assay. The results shown are obtained in one representative experiment (Fig. 1). Without induction, *CYP3A4* promoter activity in cBAL119 cells was significantly increased (3.6 fold) compared to HepG2 cells.

CYP3A4 promoter activity was significantly higher in cBAL119 cells *versus* HepG2 cells upon addition of PXR inducers rifampicin, dexamethasone and UDCA. The effects were most pronounced with rifampicin. In cBAL119 cells, addition of rifampicin significantly increased *CYP3A4* promoter activity compared to uninduced cBAL119 cells (130-fold), and induced HepG2 cells (32-fold).

These results show that *PXR* overexpression increased both basal and inducible CYP3A4 promoter activity, with rifampicin as the most potent inducer.

HepG2 cells and cBAL119 cells proliferate inside the BAL during culture

To determine bioactive mass, several parameters were analyzed in 4 isolates of both HepG2 and cBAL119 cells (Table 3). Cell pellet volume, DNA and protein levels per million cells were not significantly different in cBAL119 cells compared to HepG2 cells, indicating no effect of *PXR* overexpression on cell volume, DNA and protein content per cell. We observed a high correlation between DNA and protein levels within different isolates of either HepG2 ($r = 0.98$) or cBAL119 ($r = 1.00$).

Subsequently, proliferation of both cell lines during BAL culture was calculated by measuring total DNA content at the time of loading and after 4 days of culture. DNA content increased 1.4-fold for HepG2-AMC-BALs, and 1.5-fold for cBAL119-AMC-BALs in 4 days of culture (Fig. 2). The difference between the two cell lines was not significant, indicating no differences in proliferation capacity between HepG2 cells and cBAL119 cells in the BAL under the given conditions.

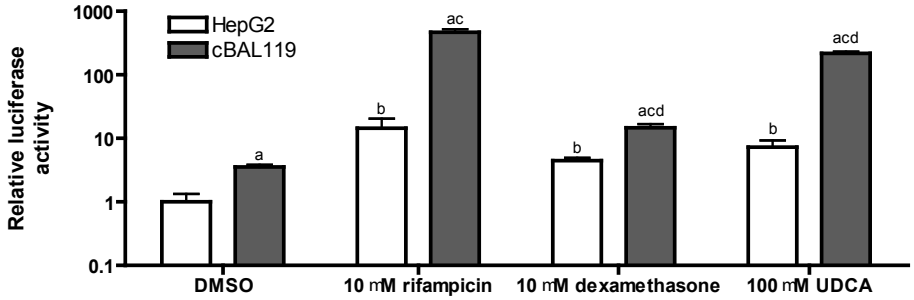

Figure 1. CYP3A4 promoter activity of HepG2 cells and cBAL119 cells using a luciferase reporter gene assay. *CYP3A4* promoter activity was normalized to uninduced HepG2 cells Significance: $^{a}p < 0.05$ cBAL119 *versus* HepG2 w/ the same inducer, $^{b}p < 0.05$ HepG2 w/ inducer *versus* HepG2 w/ DMSO (= vehicle), $^{c}p < 0.05$ cBAL119 w/ inducer *versus* cBAL119 w/ DMSO, $^{d}p < 0.05$ cBAL119 w/ dexamethasone or UDCA *versus* cBAL119 w/ rifampicin. Abbreviations: DMSO, dimethyl sulfoxide; UDCA, ursodeoxycholic acid.

Table 3. Quantification of bioactive mass in HepG2 cells and cBAL119 cells.

	Cell pellet volume ($\mu L \cdot 10^6$ cells^{-1})	AST ($U \cdot 10^6$ cells^{-1})	LDH ($U \cdot 10^6$ cells^{-1})	Protein ($\mu g \cdot 10^6$ cells^{-1})	DNA ($\mu g \cdot 10^6$ cells^{-1})
HepG2	4.20 ± 1.04	1.77 ± 0.48	5.28 ± 0.62	67.1 ± 20.9	226.5 ± 77.7
cBAL119	3.76 ± 0.90	1.69 ± 0.84	4.75 ± 1.63	52.8 ± 22.4	177.4 ± 85.6

No significant differences were observed between HepG2 cells and cBAL119 cells. Values are expressed as means ± standard deviations. Abbreviations: AST, aspartate aminotransferase; LDH, lactate dehydrogenase.

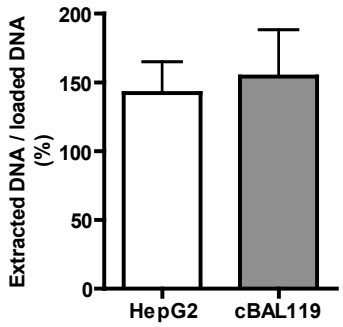

Figure 2. Proliferation during BAL culture as determined by increase in total DNA content. Values are expressed as means ± standard deviations. Differences were not significant.

Cellular integrity is maintained during BAL culture in both HepG2-AMC-BALs and cBAL119-AMC-BALs

Firstly we assessed the total AST and LDH content per million cells in samples of the cell suspension that were loaded into the BALs (Table 3). Then, to analyze cell integrity during BAL culture, we determined the AST and LDH release in both BAL groups. Enzymes levels did not significantly differ between HepG2 cells and cBAL119 cells (Fig. 3A and 3B).

In relation to the total AST and LDH content, release rates were very low for both groups during the whole culture period. In cBAL119-AMC-BALs, the maximum AST release rate was 2.23 ± 1.32 U h^{-1} g of protein^{-1}, and the maximum LDH release rate was 5.59 ± 1.65 U h^{-1} g of protein^{-1}. This corresponds with a negligible loss of approximately 0.3-0.4% of the total cell mass per BAL per day. In addition, enzyme leakage was generally highest at day 1, possibly reflecting cell damage as a consequence of the isolation procedure.

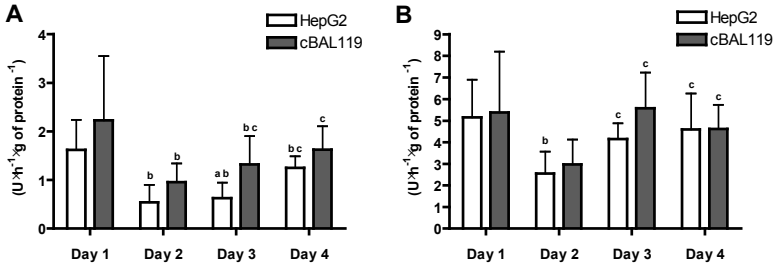

Figure 3. Cellular integrity analysis in HepG2-AMC-BALs and cBAL119-AMC-BALs. (A) AST release. (B) LDH release. Values are expressed as means ± standard deviations. Significance: $^{a}p < 0.05$ cBAL119 *versus* HepG2 on the same day, $^{b}p < 0.05$ denoted day *versus* day 1 for the same cell line, $^{c}p < 0.05$ denoted day *versus* day 2 for the same cell line. Abbreviations: AST, aspartate aminotransferase; LDH, lactate dehydrogenase.

6β-Hydroxytestosterone production rate is increased in cBAL119-AMC-BALs

We determined the production rate of 6β-hydroxytestosterone as a parameter for detoxification function in both HepG2-AMC-BALs and cBAL119-AMC-BALs. At any tested day over the 4-day culture period, cBAL119-AMC-BALs produced significantly more 6β-hydroxytestosterone than HepG2-AMC-BALs (Fig. 4). At day 1, the 6β-hydroxytestosterone production rate in cBAL119-AMC-BALs reached 0.95 μmol h^{-1} g of protein^{-1}, 1.6% of the level produced by primary human hepatoyctes in monolayer cultures (data not shown). In addition, the largest difference between HepG2-AMC-BALs and cBAL119-AMC-BALs was also observed at day 1, with a 3.8-fold higher production rate in cBAL119-AMC-BALs. From day 1 to 2, the 6β-hydroxytestosterone production rate in cBAL119-AMC-BALs decreased, and starting from day 2 there was a trend towards an increase in production rate in cBAL119-AMC-BALs. In HepG2-AMC-BALs, the 6β-hydroxytestosterone production rate remained stable throughout the culture period.

As an additional parameter for detoxification, we analyzed the conversion rate of BFC to HFC in both cell lines on the second day of BAL culture. Although there was a trend towards a higher production rate in cBAL119-AMC-BALs *vs* HepG2-AMC-BALs (46.6 ± 19.8 and 56.2 ± 7.5 nmol·h^{-1}·g of protein^{-1} respectively), this difference was not significant ($p = 0.146$).

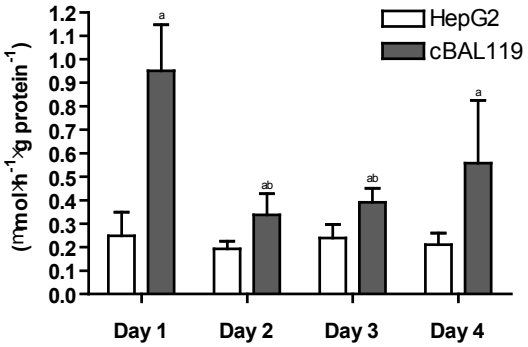

Figure 4. 6β-Hydroxytestosterone production in HepG2-AMC-BALs and cBAL119-AMC-BALs. Values are expressed as means ± standard deviations. Significance: $^{a}p < 0.05$ cBAL119 *versus* HepG2-AMC-BAL on the same day, $^{b}p < 0.05$ denoted day *versus* day 1 for the same cell line.

Functions other than detoxification differ marginally between cBAL119- and HepG2-AMC-BALs

As a marker for synthetic function, we determined the ApoA-1 production of the HepG2 and cBAL119-AMC-BALs. Over the whole culture period, the ApoA-1 production rate in cBAL119-AMC-BALs was not significantly different from HepG2-AMC-BALs (Fig. 5A). However, compared to HepG2-AMC-BALs, the ApoA-1 production rate in cBAL119-AMC-BALs was significantly higher at day 1 and 3. In both HepG2-AMC-BALs and cBAL119-AMC-BALs, the ApoA-1 production rate significantly decreased during culture.

The urea production rate in cBAL119-AMC-BALs was not significantly different from HepG2-AMC-BALs (Fig 5B). In cBAL119-AMC-BALs, the urea production rate decreased significantly from day 2 to 3, to increase again from day 3 to 4.

During BAL culture, the glucose consumption rate increased approximately two-fold between days 1 and 4 (significant) in both HepG2-AMC-BALs and cBAL119-AMC-BALs, with no significant differences between the two BAL groups (Fig 5C). Between both BAL groups, the lactate production rate was similar and increased during culture (Fig 5D). For both groups this increase was approximately two-fold between days 1 and 4.

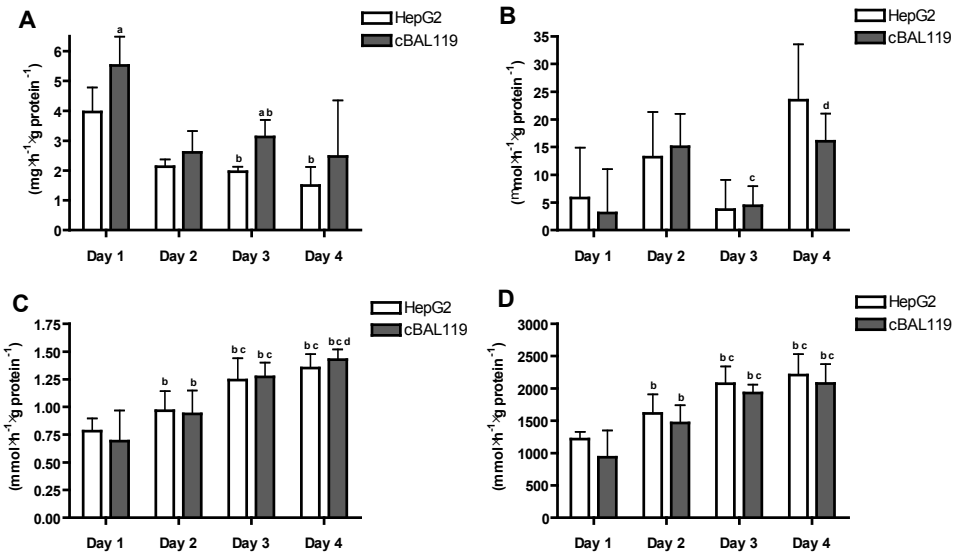

Figure 5. Non-detoxification functions of HepG2-AMC-BALs and cBAL119-AMC-BALs. (A) ApoA-1 production. (B) Urea production. (C) Glucose consumption. (D) Lactate production. Values are expressed as means ± standard deviations. Significance: [a]$p < 0.05$ cBAL119 *versus* HepG2 on the same day, [b]$p < 0.05$ denoted day *versus* day 1 for the same cell line, [c]$p < 0.05$ denoted day *versus* day 2 for the same cell line, [d]$p < 0.05$ denoted day *versus* day 3 for the same cell line.

DISCUSSION

In the present study we created the cell line cBAL119 by stably overexpressing the human *PXR* gene in HepG2 cells. We compared the cBAL119 cells with the parental HepG2 cells on various levels in both monolayer cultures as in BAL cultures. Here we provide evidence that stable overexpression of *PXR* in HepG2 cells increases the potential of these cells for BAL application. This is based on the following observations in cBAL119: 1) an increase in expression levels of several important hepatic genes involved in detoxification, 2) an increase in *CYP3A4* promoter activity, 3) an increase in detoxification activity, as shown by an increase in the rate of 6β-hydroxylation of testosterone, and 4) maintenance of proliferation capacity and of liver cell functionality, as tested by DNA analysis and several general cellular and hepatic functions.

Given the xenogenic risks related to the use of animal hepatocytes in BALs, the success of clinical application of BAL treatment is highly dependent on the availability of a human cell line possessing high hepatic functionality. An effective hepatic detoxification system is one of the prerequisites of such a cell line. This is supported by the observation of Wang *et al.* who showed that treatment with a BAL filled with HepG2 cells overexpressing *CYP3A4* increased

survival of dogs with ALF.[9] Besides CYP3A4 activity however, effective detoxification of endo- and exotoxins also depends on import, subsequent conjugation and canalicular export systems. For example, during the detoxification of high concentrations of acetaminophen, the initial hydroxylation by CYPs generates the very toxic N-acetyl-p-benzo-quinone imine, and it is only by the subsequent conjugation to glutathione and excretion, that the molecule is detoxified. Therefore, overexpressing *PXR*, as a regulator of detoxification on every level (import, hydroxylation, conjugation, and export), may be a more promising approach to increase detoxification function. We used a third generation lentiviral vector of which genes encoding structural lentiviral proteins are only expressed in the vector producing cells and not in the target cells. Hence, the risk of possible replication and/or infection of the patients treated with cBAL119-AMC-BALs is negligible.[20, 28]

We showed a 29-fold higher expression of *PXR* in cBAL119 compared to HepG2 cells, which is indicative of a successful overexpression. As a result, the cBAL119 cells showed increased basal and inducible transcript levels of *CYP3A4*, *CYP3A5* and *CYP3A7*. The transcript level of *CYP3A4* remained relatively low, and not inconceivably, this may be related to the low expression of *CAR*. However, the homology between *CYP3A4* and other family members *CYP3A5* and *CYP3A7* is almost 90%, and as a consequence CYPs within one family share many substrates.[29] Therefore, the activity of CYP3A5 and CYP3A7 may compensate for the lack in CY3A4 activity. In addition, it should be noted that CYP3A7 is principally a fetal CYP3A member, which explains the high transcript levels compared to adult liver.[30]

CYP3A4 promoter activity in basal, *i.e.* uninduced, cBAL119 cells was 3.6-fold increased compared to uninduced HepG2 cells. Tirona *et al.* reported a 1.4-fold increase in this respect.[15] Upon induction by rifampicin, *CYP3A4* promoter activity in cBAL119 cells increased further, to 130-fold the level of uninduced cBAL119 cells, and 465-fold the level of uninduced HepG2 cells. In contrast, Tirona *et al.* reported a 43-fold increase in *CYP3A4* promoter activity in their *PXR* transfected cells upon induction with rifampicin. In another study, Luo *et al.* found an increase in *CYP3A4* promoter activity of approximately 20-fold upon induction of rifampicin in their *PXR* transfected HepG2 cells.[14] Thus, *CYP3A4* promoter activity in cBAL119 cells is markedly higher than the *CYP3A4* promoter activity found in both studies mentioned above.

In addition, HepG2 cells transiently overexpressing *PXR* are not suitable for BAL application, as a stable cell line is a prerequisite for BAL application. In this regard, only one study reported about HepG2 cells stably overexpressing the *PXR* gene (DPX-2 cells). In this study *CYP3A4* promoter activity in rifampicin induced DPX-2 cells was increased 30-fold compared to uninduced DPX-2 cells.[17] This increase is also markedly less than the 130-fold increase we observed.

Detoxification on a functional level, as measured by the 6β-hydroxylation rate of testosterone was significantly higher in cBAL119-AMC-BALs compared to HepG2-AMC-BALs on every day of culture. At day 1, this difference was most pronounced (almost 4-fold). Although this increase

is substantial, it is only a fraction of the increase in *PXR* transcript levels (19-fold), or *CYP3A4* promoter activity (32-fold). It cannot be excluded that other factors, like posttranscriptional regulation of CYPs, including for instance cytochrome P450 oxidoreductase activity, and transport processes which are not controlled by PXR, are rate limiting. After day 1 of culture, testosterone hydroxylation in cBAL119-AMC-BAL declined. At day 2, metabolic rates were at their minimum, and from day 2 to 4, we observed a trend towards an increase in testosterone hydroxylation. This pattern may be associated with the organization and hepatic differentiation of the cells. Probably, the cells still showed the monolayer phenotype at protein level at day 1, but the isolation procedure and resulting loss of cell-cell contacts lead to diminished *CYP3A* expression on day 2. Greuet *et al.* have previously shown that cell-cell contacts are a prerequisite for high *CYP3A4* expression and activity in primary human hepatocytes.[31] The increase in testosterone hydroxylation starting from day 2 may be explained by a gradual restoration of cell-cell contacts and/or by cell proliferation. Interestingly, the absence of these trends in HepG2 cells, while the growth rate of cBAL119 and HepG2 was similar, suggests that the effect of cell organization on the 6β-hydroxylation of testosterone is PXR mediated. The 6β-hydroxylation rate of testosterone in cBAL119-AMC-BALs reached approximately 1.6% of the rate of primary human hepatocytes in monolayers cultures. Although not substantial, it is unknown whether this amount is sufficient in terms of treating ALF patients with cBAL119-AMC-BALs. To test this hypothesis, cBAL119-AMC-BALs will have to be compared with HepG2-AMC-BALs in an animal model of ALF.

As an additional parameter for CYP activity, we also studied BFC conversion rates in BALs loaded with both cell lines at the second day of culture. Conversion of BFC to HFC is predominantly catalyzed by CYP1A1, CYP1A2 and CYP2B1, and only to a lesser extent by CYP3A4.[26, 32, 33] Our data show a trend towards an increased conversion rate in cBAL119-AMC-BALs, but we did not find a significant difference between the two cell lines. Possible this is related to a lack of upregulation of *CYP1A1*, *CYP1A2* and *CYP2B1* in cBAL119 cells. *CYP2B1* is not a target gene of PXR in humans, supporting this hypothesis.[13] In contrast, CYP1A1 and CYP1A2 have been described as target genes for PXR in humans; however, we did not measure higher CYP1A2 mRNA levels in cBAL119 cells compared with HepG2 cells (data not shown). This could also underlie the lack of difference in HFC production between the two cell lines.

The cBAL119 and HepG2 cells exhibited similar growth rates, urea production, lactate production and glucose consumption, as well as similar levels of AST and LDH leakage. However, the transcript levels of *albumin* and the ApoA-1 production rate both significantly increased in cBAL119 cells, indicating that hepatic synthetic function is increased in cBAL119. This is in agreement with recent experiments that demonstrate that the activity of PXR is not restricted to detoxification. PXR exerts its activity also on energy metabolism, binds to promoters of genes implicated in other processes like apoptosis and electron transport, and indeed the *APOA1* gene has previously been identified as a target of PXR.[34-36]

Unfortunately, the urea production rate was low in both cell lines when cultured in the AMC-BAL, being approximately 10% of the rate of a BAL filled with primary porcine hepatocytes. Moreover, Damelin *et al.* demonstrated that urea production in HepG2 cells does not originate from exogenously added ammonia, as the urea cycle is non-functional in HepG2 cells. Instead, urea production is mainly due to intramitochondrial arginase II activity.[37] Our data support these findings, as ammonia elimination was negligible in both HepG2 and cBAL119-AMC-BALs (data not shown), and the urea cycle genes *CPS* and *ARG1* were very low expressed in both cell lines.

As clinical application of liver support systems relying on cell lines is dependent on the stability of these cell lines, it would be interesting to investigate whether the functionality of cBAL119-AMC-BALs will further improve during prolonged culture. In general, our data show that the cBAL119-AMC-BALs demonstrated the highest hepatic functionality on day 1, a decrease on day 2, and subsequently a regain of function on days 3 and 4. As discussed, cellular organization related differentiation could underlie this phenomenon, and it is therefore not inconceivable that this gain in functionality will continue during prolonged culture. Additional studies on cBAL119-AMC-BALs will be necessary to address this question.

In conclusion, our data demonstrate that stable overexpression of *PXR* in HepG2 cells improves their detoxification function, with conservation of other hepatic functions and proliferation capacity. As detoxification plays a central role in the treatment of ALF, *PXR* overexpression in HepG2 cells increases the potential of this cell line for BAL application. However, improving detoxification will not necessarily translate in increased survival when testing these cells an animal model of ALF. In addition, further research is needed to either improve the ammonia elimination and urea production capacity of the cBAL119 cells, or alternatively, investigate the possibility of combining a cBAL119-AMC-BAL with an additional ammonia detoxification device. Subsequently, such a system should show proof of principle in an animal model of ALF, before a phase 1-2a study in patients with ALF can be started.

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CHAPTER 4

The HepaRG cell line is suitable for bioartificial liver application

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ABSTRACT

Background: For bioartificial liver application, cells should meet the following minimal requirements: ammonia elimination, drug metabolism and blood protein synthesis.

Aim: Here we explore the suitability of HepaRG cells, a human cell line reported to differentiate into hepatocyte clusters and surrounding biliary epithelial-like cells at high density and after exposure to dimethyl sulfoxide (DMSO). The effect of carbamoyl-glutamate (CG), an activator of urea cycle enzyme carbamoylphosphate synthetase (CPS) was studied additionally.

Methods: The effects of DMSO and/or CG were assessed in presence of $^{15}NH_4Cl$ on HepaRG cells in monolayer. We tested hepatocyte-specific functions at transcript and biochemical level, cell damage parameters and performed immunostainings.

Results: Ureagenesis, ammonia/galactose elimination and *albumin*, *glutamine synthetase* and *CPS* transcript levels were higher in -DMSO than +DMSO cultures, probably due to a higher cell content and/or cluster-neighbouring regions contributing to their functionality. DMSO treatment increased *cytochrome P450 (CYP)* transcript levels and CYP3A4 activity, but also cell damage and repressed hepatic functionality in cluster-neighbouring regions. The levels of ammonia elimination, apolipoprotein A-1 production, and transcript levels of *CYP3A4*, *CYP2B6* and *albumin* reached those of primary hepatocytes in either the + or -DMSO cultures. Preconditioning with CG increased conversion of $^{15}NH_4Cl$ into ^{15}N-urea 4-fold only in -DMSO cultures.

Conclusions: Hence, HepaRG cells show high metabolic and synthetic functionality in the absence of DMSO, however, their drug metabolism is only high in the presence of DMSO. An unparalleled broad hepatic functionality, suitable for bioartificial liver application, can be accomplished by combining CG treated -DMSO cultures with +DMSO cultures.

INTRODUCTION

Bioartificial livers (BALs) have been developed to temporarily support patients with severe liver failure.[1] BALs are based on a bioreactor with functional liver cells, ideally human proliferative cells with hepatic function. This human bio-component should detoxify accumulating toxic compounds via the cytochrome P450 (CYP) system, maintain metabolic homeostasis and synthesize blood proteins. An important function is the elimination of ammonia, a neurotoxin accumulating during severe liver failure.[2] The liver removes ammonia primarily through the urea cycle and secondarily through fixation into amino acids, particularly glutamine by glutamine synthetase (GS).

Frequently HepG2 cells have been applied as human proliferative bio-component in BALs.[3] Although HepG2 and subclone C3A show hepatic functionality, like synthesis of blood proteins, the urea cycle is not functional, resulting in absence of ammonia detoxification via this route.[4, 5] Notably, urea production solely reflects arginase (ARG) activity. Moreover, the ammonia-eliminating capacity of HepG2 cells via glutamine synthesis is also marginal and even ammonia production has been reported.[6, 7] Furthermore, the HepG2 cells lack CYP enzymes, except fetal isoforms.[8] The findings for HepG2 are exemplary for most, if not all, human bio-components applied to BALs so far.

In 2002, Gripon et al. reported cell line HepaRG, which approximates primary human hepatocytes (PHHs) in various hepatic functions after culturing for 14 days in HepaRG medium with 2% dimethylsulfoxide (DMSO), preceded by a 14-days proliferation phase without DMSO.[9] The HepaRG cultures progress during that 28-day period from a progenitor phenotype into a heterogeneous culture with hepatocyte-like clusters representing 50-55% of the total cell population and neighbouring cells that express biliary epithelial markers.[10] A recent microarray analysis showed that HepaRG cells are more related to human liver and PHHs than HepG2 cells.[11] For most drug-metabolizing genes, including *CYP2B6* and *CYP3A4*, expression levels are associated with the presence of DMSO, possibly via upregulation of nuclear hormone receptors constitutive androstane receptor (CAR) and pregnane X receptor (PXR).[12, 13] However, DMSO seems ineffective in upregulating the synthesis of blood proteins, like albumin (ALB). Yet, many functions relevant to BAL application and their response to DMSO treatment have not been investigated. Hence, we investigated the effect of DMSO treatment on HepaRG functionality in the context of a BAL, with a focus on nitrogen metabolism. Furthermore, we improved the ureagenesis of HepaRG cells using carbamoyl-glutamate (CG), an analogue of N-acetylglutamate, the physiological allosteric activator of carbamoylphosphate synthetase (CPS), the rate-determining enzyme of the urea cycle under normal physiological conditions.[14]

METHODS

Cell culture

HepaRG cells were kindly provided by prof. C. Guguen-Guillouzo (INSERM, Rennes). HepaRG cells were cultured in 24-well culture plates (Corning, New York) as described.[9] Analyses were conducted on -DMSO and +DMSO cultures, defined as HepaRG cultures 28 days post-seeding, with the last 2 weeks cultured either in absence or presence of 2% DMSO (Sigma, St. Louis), respectively. The effect of CG was tested by adding 1 mM CG (Sigma) either during the test and/or during 5 days preceding the test.

As reference material, small liver samples and PHHs were isolated from liver tissue of patients undergoing partial hepatectomy as described.[15] The procedure was in accordance with the ethical standards of the institutional committee on human experimentation and after informed consent. The PHHs were seeded at a density of 0.25 million/cm^2 in 24-well plates in HepaRG or WE medium. After 4 hours attachment, the culture medium was refreshed.[15]

Reverse transcription-polymerase chain reaction (RT-PCR)

RNA was isolated by using the RNeasy mini kit (Qiagen, Venlo). As a reference, two human liver samples were included in the analyses. cDNA was generated using gene-specific RT primers.[16] Real-time PCR was performed as described.[17] Transcript levels were calculated, normalized for 18S ribosomal RNA, and expressed as a percentage of the mean of the two human liver samples.[18] Primer sequences and amplicon sizes are depicted in Supplemental Table 1.

Hepatocyte function tests

Hepatocyte function tests were performed on HepaRG cultures and, for comparison, on PHHs at day 1 or 2 post-seeding. Most of the tested liver functions were optimal and stable during that period, however for CYP3A4 activity assays, PHHs were only used as a reference at 1 day post-seeding. After washing the cultures twice with phosphate-buffered saline (PBS, Fresenius Kabi GmbH, Graz) culture medium was replaced by 1 mL of test medium (HepaRG medium with 1.5 mM $^{15}NH_4Cl$ (Sigma), 2.27 mM *D*-galactose (Sigma), 2 mM *L*-lactate (Sigma) and 2 mM ornithine hydrochloride (Sigma)). Medium samples were taken after 45 min and 24 hours of incubation. For determination of CYP3A4 activity, cultures were incubated with HepaRG medium including 60 μM 3-[(3,4-difluorobenzyl)oxy]-5,5-dimethyl-4-[4-(methylsulfonyl) phenyl] furan-2(5*H*)-one (DFB), kindly provided by Dr. Nicoll-Griffith, Merck Frosst, Canada Limited, Kirkland, and samples were taken after 0.5, 1 and 2 hrs. Finally, all test cultures were washed twice with PBS and stored at -20°C for protein determination.[19]

Biochemical assays

Concentrations of ammonia, urea, apolipoprotein A1 (APOA1), protein and activities of aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) were measured in the test samples as reported.[17] Singly and doubly labeled ^{15}N-urea were measured using gas chromatography-mass-spectrometry.[20] Amino-acid concentrations were assessed by gradient reversed-phase high-performance liquid chromatography.[21] For CYP3A4 activity, the concentration of 3-hydroxy-5,5-dimethyl-4-[4-(methylsulfonyl)phenyl]furan-2(5*H*)-one (DFH), the fluorescent metabolite of DFB, was determined as described [19] after a 2h-treatment with β-glucuronidase/arylsulfatase (Roche, Basel) (150 Fishman units/mL and 1200 Roy units/mL, respectively) at 37°C. .Production rates were established by calculating the concentration changes in time and were optionally corrected for protein content/well.

Immunocytochemistry

The cultures were fixed in a 40% methanol (Merck, Darmstadt), 40% acetone (Merck), and 20% water mixture (v/v/v) and stained for GS as described.[16] Similarly, CPS was visualized using a rabbit anti-CPS antibody (1:500,) and alkaline phosphatase-labeled goat anti-rabbit IgG 170-6518 (1:200, Bio-Rad, Hercules). ALB was visualized using goat anti-ALB antibody (1:200, Sigma) and alkaline phosphatase-labeled rabbit anti-goat IgG (1:50, Sigma).[22] The reactions were developed with 5-bromo-4-chloro-3'-indolyphosphate p-toluidine and nitro-blue tetrazolium chloride (Roche). In addition, the cultures were evaluated by immunofluorescence for simultaneously staining of CYP3A4 and GS. CYP3A4 was visualized using a rabbit anti-human CYP3A4 antibody (1:100, Fitzgerald Industries International, Acton) and Alexa Fluor 488 (cyan-green)-labeled goat anti-rabbit IgG (1:1000, Molecular Probes, Eugene). GS was visualized using a mouse anti-GS antibody (1:500, Transduction Laboratories, Lexington) and Alexa Fluor 594 (orange-red)-labeled goat anti-mouse IgG (1:1000, Molecular Probes). In addition, cultures were embedded in Vectashield (Vector Laboratories, Burlingame) containing 4,6-diaminidino-2-phenylindole (DAPI) to counterstain DNA. The number of DAPI-stained nuclei was counted manually in ten fields of 4 different photographs of +DMSO and -DMSO cultures. Negative controls did not include the primary antibody incubation.

Statistical analysis

Student's t-tests were used to determine statistical differences. Significance was reached if $P < 0.05$. SPSS 12.0.1 (SPSS Inc., Chicago) was used for statistical analysis. Average values (± standard deviation) are reported.

RESULTS

Effect DMSO treatment on expression profile

The -DMSO and +DMSO HepaRG cultures were assessed for expression of various hepatic genes (Table 1). Most genes investigated were unaffected by DMSO treatment. However, as previously reported, DMSO treatment substantially increased the transcript levels of *CYP1A2*, *CYP2B6*, and *CYP3A4*[12] This increase was not associated with increased mRNA levels of transcription factors hepatocyte nuclear factor 4 alpha (HNF4A), PXR and CAR. In contrast, the mRNAs of blood proteins ALB, transferrin (TF), and alpha-fetoprotein (AFP) were significantly decreased, as well as GS and, most dramatically, CPS. Compared to human liver, the transcript levels of the HepaRG cultures varied to great extent: from low levels (≤31%) for *CYP1A2*, *OATP2*, *CPS*, *OTC*, and *ARG1* to high levels (≥154%) for *MRP2*, *ARG2*, and *AFP*.

Table 1. Transcript levels of -DMSO and +DMSO cultures.

Gene	Description	-DMSO	+DMSO	Fold change +DMSO	*P* value
	Transcription factor				
HNF4A	Hepatocyte nuclear factor 4 alpha	88 ± 89	61 ± 21	0.7	ns
PXR	Pregnane X receptor	44 ± 16	42 ± 19	0.9	ns
CAR	Constitutive androstane receptor	15 ± 12	27 ± 15	1.8	ns
	Detoxification				
CYP1A2	Cytochrome P450 1A2	0.1 ± 0.1	0.6 ± 0.2	6.0	0.007
CYP2B6	Cytochrome P450 2B6	3 ± 1	61 ± 30	20.3	0.001
CYP3A4	Cytochrome P450 3A4	10 ± 8	69 ± 47	6.9	0.008
NTCP	Sodium/bile acid cotransporter	11 ± 2	16 ± 11	1.5	ns
OATP2	Organic anion transporter protein 2	12 ± 10	7 ± 6	0.6	ns
MRP2	Multidrug resistance protein 2	154 ± 94	240 ± 72	1.6	ns
	Metabolism				
CPS	Carbamoylphosphate synthetase	31 ± 19	1.1 ± 0.4	0.04	0.015
OTC	Ornithine transcarbamoylase	6 ± 5	14 ± 9	2.3	ns
ASS	Argininosuccinate synthetase	81 ± 32	80 ± 22	1.0	ns
ASL	Argininosuccinate lyase	56 ± 17	57 ± 16	1.0	ns
ARG1	Arginase 1	15 ± 11	5 ± 4	0.3	ns
ARG2	Arginase 2	571 ± 364	718 ± 463	1.3	ns
GS	Glutamine synthetase	214 ± 66	90 ± 29	0.4	0.014
	Blood protein				
ALB	Albumin	66 ± 24	23 ± 4	0.3	0.007
TF	Transferrin	110 ± 34	43 ± 24	0.4	0.018
AFP	Alpha-fetoprotein	294 ± 52	158 ± 41	0.5	0.006

Transcript levels are indicated as % of mean mRNA levels of two human liver samples and normalized for 18S ribosomal RNA. Values are given as mean ± SD (n=5). The fold changes in transcript levels due to DMSO treatment are indicated. *P* values refer to -DMSO *vs* +DMSO cultures. Abbreviation: ns, not significant.

Effect DMSO treatment on total protein and hepatic functionality

The DMSO treatment resulted in a 56% lower protein/well content in +DMSO cultures *versu*s -DMSO cultures and increased the cell leakage markers AST and LDH (normalized for total protein/well) 3- to 4-fold (Table 2). The number of nuclei was 48% reduced in +DMSO cultures compared to -DMSO cultures, which corresponds grossly to the 56% reduction in total protein content/well (Fig. 3). However, the protein-normalized ammonia elimination and urea production rates were only 32%-33% higher in the +DMSO cultures, yielding absolute ammonia elimination and urea production rates, uncorrected for total protein, 1.5-fold higher in -DMSO cultures compared to +DMSO cultures. The protein-normalized APOA1 production was not significantly affected by DMSO treatment. DMSO-treated HepaRG cells eliminated ammonia at 113%, produced urea at 8.4% and APOA1 at 174% when compared to PHHs. These rates were 26%-40% lower in -DMSO HepaRG cultures. The protein-normalized galactose elimination was 2-fold higher in the -DMSO cultures compared with the +DMSO cultures and reached 17% of the rate of PHHs. Finally, protein-normalized DFH production, as a marker for CYP3A4 activity, was 3.7-fold higher in +DMSO HepaRG cultures respective to -DMSO cultures, reaching 19% of the activity of PHHs.[19]

Table 2. Characteristics of -DMSO and +DMSO cultures (n≥10 from ≥3 independent experiments).

	Parameter	-DMSO	+DMSO	Fold change +DMSO	*P* value	PHH
Absolute values	Total protein (μg/well)	247 ± 47	109 ± 21	0.4	<0.001	
	Ammonia elimination (nmol/h)	17.0 ± 4.6	11.6 ± 4.3	0.7	<0.001	
	Urea production (nmol/h)	1.2 ± 0.5	0.8 ± 0.5	0.7	0.003	
	Galactose elimination (nmol/h)	26 ± 5.4	7.2 ± 1.8	0.3	<0.001	
	APOA1 production (μg/h)	0.17 ± 0.11	0.11 ± 0.11	0.6	ns	
	DFH production (nmol/h)	0.34 ± 0.21	0.75 ± 0.11	2.7	<0.001	
	AST leakage (mU/h)	0.27 ± 0.07	0.43 ± 0.08	1.6	<0.001	
	LDH leakage (mU/h)	1.54 ± 0.24	1.87 ± 0.43	1.2	<0.001	
Protein normalized values	Ammonia elimination (nmol/h/mg protein)	72 ± 21	107 ± 41	1.5	<0.001	95 ± 5.0
	Urea production (nmol/h/mg protein)	5.0 ± 2.0	8.1 ± 6.8	1.6	0.02	96 ± 34
	Galactose elimination (nmol/h/mg protein)	100 ± 24	58 ± 17	0.6	<0.001	577 ± 422
	APOA1 production ((μg/h/mg protein)	0.78 ± 0.50	1.06 ± 0.95	1.4	ns	0.61 ± 0.16
	DFH production (nmol/h/mg protein)	1.0 ± 0.4	3.7 ± 0.5	4.5	<0.001	19.0 ± 5.8
	AST leakage (mU/h/mg protein)	1.3 ± 0.4	4.9 ± 2.4	3.8	<0.001	ND
	LDH leakage (mU/h/mg protein)	7.4 ± 2.2	21.2 ± 9.4	2.8	<0.001	ND

Values are given as mean ± SD. The fold change in characteristics due to DMSO treatment are indicated. *P* values refer to -DMSO *vs* +DMSO cultures. As a reference, the protein normalized hepatic functions of PHHs are given. Abbreviaions: ns, not significant; ND, not determined.

Localization of *ALB, CPS and GS* expression

To investigate the higher *ALB*, *CPS*, and *GS* mRNA levels and the relatively high ammonia elimination and urea production rates in -DMSO cultures *versus* +DMSO cultures, the localization of ALB-, CPS- and GS-positive cells was determined by immunocytochemistry. Fig. 1 shows that both cultures contained clusters of cuboid cells with granular appearance surrounded by larger and flattened cells, with the clusters being more clearly delineated and granular in the +DMSO cultures. Surprisingly, the -DMSO cultures contained both positive and negative cells for ALB, CPS and GS in the hepatocyte-like clusters, as well as in the cluster-neighbouring regions. DMSO treatment repressed *ALB*, *CPS* and *GS* expression in cluster-neighbouring regions, whereas the hepatocyte clusters still contained both positive and negative cells. However, the assessment of the immunostainings of the hepatocyte clusters of +DMSO cultures was hampered by their high granularity.

To limit the background due to granularity and identify more accurately the hepatocyte clusters, we performed an immunofluorescence double staining for GS and CYP3A4 (Fig. 2). As previously observed, CYP3A4 staining was confined to the clusters of hepatocyte-like cells in both -DMSO and +DMSO cultures.[10] The GS-CYP3A4 double stainings indicated near mutually exclusive localization of both proteins in -DMSO cultures, with CYP3A4-positive cells only localized in the hepatocyte clusters and GS-positive cells in both the hepatocyte clusters and the cluster-neighbouring regions. The DMSO treatment led to co-expression of *GS* and *CYP3A4* in the hepatocyte clusters and to absence of GS-positive cells in the cluster-neighbouring regions. A DAPI-Cyp3A4 double staining was included to confirm that cells were present in negatively stained regions (Fig. 3). The results indicate that the higher *ALB*, *CPS* and *GS* transcript levels in -DMSO cultures *versus* +DMSO cultures may, at least partly, be explained by differences in localization of expression. Furthermore, hepatic function in -DMSO cultures is not exclusively confined to the hepatocyte-like clusters, but is also present in the cluster-neighbouring cells.

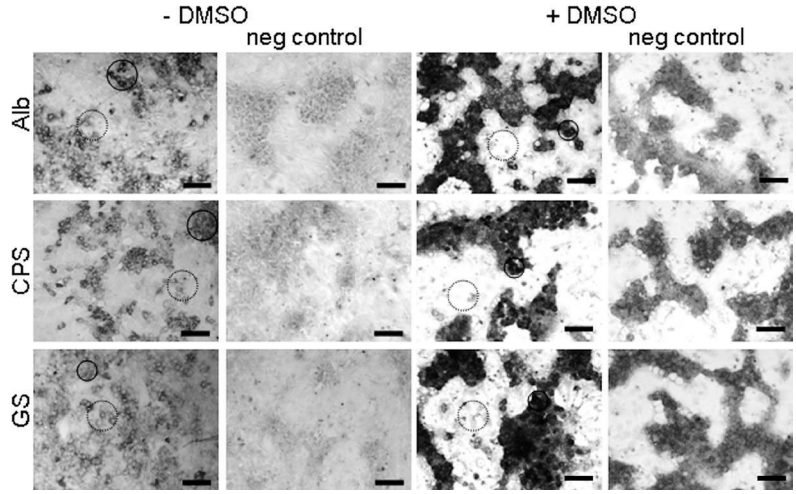

Figure 1. Immunostainings for Alb, CPS and GS. Negative controls are included. Positively stained cells of hepatocyte-like clusters and cluster-neighbouring regions are indicated with straight and dotted circles, respectively. Bars represent 100 μm.

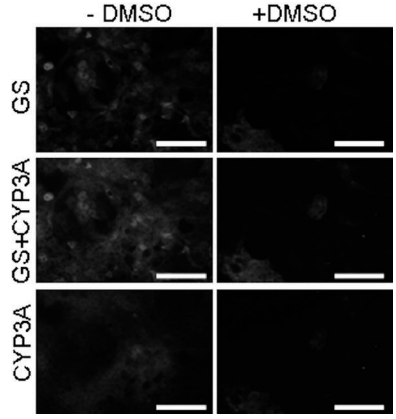

Figure 2. Immunofluorescence double stainings for CYP3A4 and GS. Bars represent 100 μm. *For color figure, see page 211.*

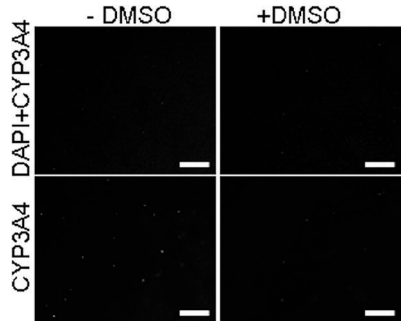

Figure 3. Immunofluorescence staining for CYP3A4 with DAPI counterstaining. Bars represent 100 μm. *For color figure, see page 211.*

Nitrogen metabolism and the effect of CG

We aimed to improve the ureagenesis by including 1 mM CG in the culture medium.[23] Although ammonia elimination remained unaffected, urea production increased two-fold upon addition of CG in the -DMSO and not in the +DMSO cultures (Fig. 4). A 5-day preconditioning period with CG was required to achieve the maximal effect in the -DMSO cultures. An instantaneous effect of CG on ureagenesis was also observed, since cultures not pretreated with CG, but tested in presence of CG displayed improved ureagenesis.

The metabolic fate of ammonia was investigated by loading the cultures with 1.5 mM $^{15}NH_4Cl$ and subsequent measuring the mass-enriched fraction of urea (Fig. 5). We detected both singly and doubly labelled urea (ratio 2:1, irrespective CG treatment), demonstrating ammonia fixation into urea occurs both via carbamoylphosphate/citrulline synthesis and via glutamate/aspartate synthesis in HepaRG cells, as in primary hepatocytes.[24] CG treatment increased conversion of $^{15}NH_4Cl$ into ^{15}N-urea 4-fold in -DMSO cultures. The ^{15}N urea enrichment in +DMSO cultures remained ~10% and was unaffected by CG treatment. Amino acids were consumed in both +DMSO and -DMSO cultures, irrespective of CG treatment, at a rate of 50 nmol/h/mg protein, so cold ammonia may have been produced by amino acid catabolism. Under optimal urea cycle activity, *i.e.* in CG-treated -DMSO cultures, the percentage of ammonia converted to urea therefore ranged from 2.4% to 8% under maximal and minimal catabolic conditions, respectively. This suggests that minimally 90% of the eliminated ammonia is fixated into amino acids. Notably, evaporation of ammonia can be excluded as explanation, since ammonia was not eliminated from empty wells.

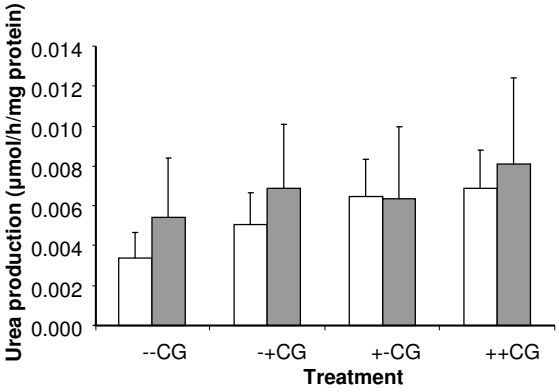

Figure 4. The effect of 1mM CG on the urea production of -DMSO (white bars) and +DMSO (gray bars) cultures. Four experimental groups were included per culture: --, no exposure to CG; -+, CG only present during test phase; +-, CG only present in preincubation phase of 5 days prior to the test phase; ++, CG present in preincubation and test phase (n=16 from 5 independent experiments). Significance within -DMSO culture: *, $p < 0.05$ -- *vs* other groups; #, $p < 0.05$ -+ *vs* +- and ++.

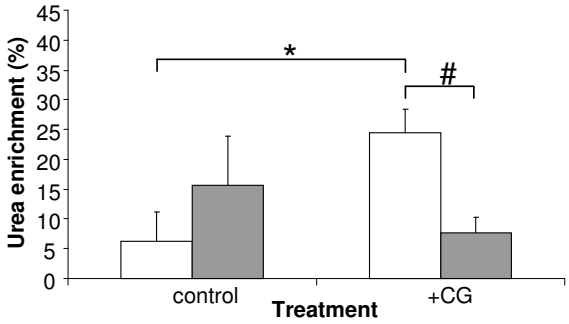

Figure 5. The effect of 1mM CG on the conversion of ^{15}N ammonia into urea by -DMSO (white bars) and +DMSO (gray bars) cultures. Two experimental groups were included per culture: control, no exposure to CG; +CG, CG present in preincubation and test phase (n=6 from 2 independent experiments). Significance: *, $p < 0.05$ within -DMSO cultures; # $p < 0.05$ between -DMSO and +DMSO cultures.

Substantial net metabolism of individual amino acids (>10 nmol/h/mg protein) during 24 hours was only found for glutamate, glutamine, alanine and leucine (Fig. 6). The -DMSO cultures consumed glutamate and produced glutamine, whereas the +DMSO cultures showed no relevant glutamate metabolism and a non-significant trend to glutamine consumption. These results may correspond to the higher *GS* expression levels in the -DMSO cultures relative to the +DMSO cultures. However, the protein-normalized ammonia elimination was higher in the +DMSO cultures. Therefore, the amino-acid metabolism might differ to a large extent between both cultures. Furthermore, both cell cultures produced alanine and consumed leucine. Although not substantial, a net consumption of arginine of 4-5 nmol/h/mg protein was measured in both -DMSO and +DMSO cultures, suggesting that ARG activity may indeed play a role in urea production. Preconditioning and testing in the presence of CG did not alter amino-acid metabolism or the expression levels of genes indicated in Table 1 (results not shown).

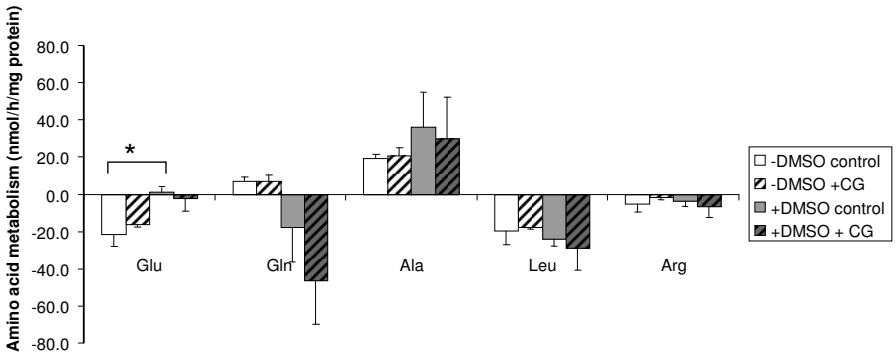

Figure 6. Tthe effect of 1mM CG treatment on the amino acid metabolism of -DMSO (white bars) and +DMSO (gray bars) cultures. Striped bars represent cultures treated with CG in preincubation and test phase. Only amino acids with substantial metabolism in one or both cultures are shown (n=6 from 2 independent experiments). Significance: *, $p < 0.05$ between -DMSO and +DMSO cultures.

DISCUSSION

This study shows that HepaRG cells display a broad array of hepatic functions, like ammonia elimination and APOA1 production, as well as gene expression levels of *CYP3A4*, *CYP2B6* and *ALB* corresponding to levels of PHHs and human liver, and also a relatively high CYP3A4 activity, which renders them suitable for BAL application. A novel dual effect of DMSO on HepaRG cultures was found, confirming a CYP-dependent stimulation of detoxification, but also the undesired inhibition of ureagenesis, ammonia/galactose elimination and *ALB*, *GS* and *CPS* expression.[12, 25] In the absence of DMSO, we could increase urea-cycle activity by preconditioning the cells with CG.

Although some functions are still limited, e.g. ureagenesis, galactose elimination and transcript levels of specific genes, the HepaRG cell line is superior to other bio-components in its coverage of the hepatic functionality spectrum.[3] Notably, the unparalleled broad functionality spectrum of HepaRG cultures can only be accomplished when combining HepaRG cultures that undergo two divergent differentiation protocols differing in exposure to DMSO.

DMSO was originally introduced as HepaRG-differentiating agent.[9] However, our results indicate that omission of DMSO results in high levels of several hepatic characteristics, including the ammonia elimination, urea production, galactose elimination and expression of *CPS*, *GS*, *ALB* and *TF*. For ammonia elimination and urea production, these differences are primarily a result of the lower cell density in the +DMSO cultures, since protein-normalized values were equal or higher in +DMSO cultures *versus* -DMSO cultures. However, the other above mentioned functions were still enhanced after normalization for total protein/well or 18S content. Our findings correspond to results of microarray analyses of 14-days -DMSO cultures and 28-days +DMSO cultures, although we used + and -DMSO cultures of equal age (28 days).[11] This study showed that pathways upregulated in +DMSO cultures relative to -DMSO cultures included a.o. xenobiotic metabolism, whereas downregulated pathways concerned a.o. developmental processes extracellular signaling, and amino-acid metabolism.

Our ALB, CPS and GS immunostainings suggest that these observations may, at least partly, be explained by differences between both cultures in the distribution of hepatic proteins among the cluster-neighbouring regions and hepatocyte-like clusters. In -DMSO cultures hepatic function is not exclusively confined to the hepatocyte-like clusters, as occurring in +DMSO cultures. Instead, in the -DMSO cultures cells within the cluster-neighbouring regions express *ALB*, *CPS* and *GS*, indicating that these cells do not display the biliary phenotype. This previously reported biliary phenotype of cluster-neighbouring regions may therefore only be elicited by DMSO.[10]

DMSO treatment eventually leads to co-localization of diverse hepatic proteins exclusively in the hepatocyte clusters. The inhibitory effect of DMSO on hepatic functionality of cluster-neighbouring cells and conversely, the stimulatory effect on hepatocyte-like clusters may be related to a differential toxicity for cluster-neighbouring and hepatocyte-like cluster cells.

Alternatively, this dual DMSO effect may be related to a differential modulation of the histone deacetylating effect of DMSO, for instance as a consequence of the differential expression profile of extracellular signalling genes between both cell types.[11, 26]

The HepaRG cell line displays an ammonia-eliminating capacity similar to that of PHHs. The low urea cycle activity and, conversely the high *GS* transcript levels suggest that ammonia elimination occurs primarily through fixation in amino acids. In -DMSO cultures, glutamate consumption coincided with glutamine production, in favour of substantial GS activity. This effect was not observed in the +DMSO cultures, which may relate to the limited sensitivity of the assay for cultures with low cell density. A high GS activity has also been observed in other human hepatocyte cell lines, *e.g.* HepG2 and cBAL111.[27, 28]

It is difficult to predict the consequences of ammonia being predominantly fixated into glutamine, rather than being converted to urea by the HepaRG cells, when clinically applied in a BAL system. Increased plasma glutamine is non-toxic for the brain, in contrast to high intracellular concentrations in the astrocyte produced by astrocytic GS from glutamate and ammonia.[29] However, metabolism of glutamine in intestine and other body sites by glutaminase, will yield again ammonia, which may contribute to the development of hepatic encephalopathy. In that respect glutamine serves only as a temporary sink of ammonia, and only the progression of hepatic encephalopathy may be diminished. Therefore, under physiological conditions, the urea-cycle is considered as the preferable route for ammonia fixation, as urea itself is not toxic and easily excreted by the kidney. However, during acute liver failure renal insufficiency often develops, urea accumulates and plasma ammonia levels may increase due to urease-producing intestinal bacteria.[30] Nevertheless, it remains an important challenge to improve urea cycle activity of the biocomponent of a BAL. This has partly been accomplished with our pretreatment with CG (see below).

The high *ARG2* transcript levels, the low *ARG1* expression, the arginine consumption and the relatively low urea enrichment data suggest that ARG2 activity most probably accounts for much of the urea production. Previously it was concluded that ureagenesis of C3A cells was solely due to ARG2 activity, as ARG1 was absent.[5] This suggests that high ARG2 activity may be a general property of degenerate hepatocyte cell lines.

As indicated, the detoxification of ammonia via the urea cycle was limited in comparison to PHHs, which may relate to low expression levels of urea cycle enzymes *OTC*, *ARG1*, and particularly in the +DMSO cultures of *CPS*. Furthermore, the increased conversion of ammonia into urea upon CG treatment in the -DMSO cultures indicates that the endogenous allosteric activator of CPS, N-acetylglutamate, is limiting the urea cycle in -DMSO cultures.[31] This may be due to low expression of *N-acetylglutamate synthase*. Inspection of the recently reported microarray database revealed that the *N-acetylglutamate synthase* transcript level is indeed 30-fold lower in HepaRG cells compared to human liver.[11] CG treatment increased the conversion of ammonia into urea 4-fold only in the -DMSO cultures, demonstrating that its supplementation

is only effective if the number of CPS-positive cells is also increased. Together these results suggest that the urea cycle in -DMSO cultures is principally limited by N-acetylglutamate levels, whereas in +DMSO cultures, it is primarily limited by the low level of *CPS* expression. The novel preconditioning effect of CG might become relevant for clinical BAL application. Namely, after culturing the HepaRG cells in the presence of CG, no additional supply of CG will be necessary during patient treatment.

In conclusion, the HepaRG cell line is a promising cell line for BAL application. However, to achieve broad hepatic functionality, it may be necessary to use a combination of two different bioreactors with either +DMSO or -DMSO treated HepaRG cells. In addition CG treatment of -DMSO cells prior to connection to the patient will increase urea synthetic capacity of the BAL and may improve patient outcome.

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Supplemental Table 1. Primer sequences used in the RT-PCR analyses with resulting amplicon sizes.

Gene	RT primer	Sense primer	Antisense primer	Amplicon size (bp)
18S rRNA	CGAACCTCCGACTTTCGTTT	TTCGGAACTGAGGCCATGAT	CGAACCTCCGACTTTCGTTCT	151
AFP	CGTTTTGTCTTCTCTTCCCC	TKCCAACAGGAGGCYATGC	CCCAAAGCAKCACGAGTTTT	306
ALB	ACTTCCAGAGCTGAAAAGCATGGTC	TGAGCAGCTTGGAGAGTACA	GTTCAGGACCACGGATAGAT	189
ARG1	TGTGATTACCCTCCCGAGCAAGTC	TTGGCAAGGTGATGGAAGAAACA	CCTCCCGAGCAAGTCCGAAACAA	305
ARG2	ACAAGGGCAGAAAAGAAAAGGAGT	GGTCCCGCTGCCATAAGAGA	GGCATCAACCCAGACAACACAA	299
ASL	CTGCAGTGACAGCTGGTTGAGG	TCAACTTTGGGGCCATCACTCTCA	CGGGGTTTTTCTTCTGGGGCATCA	212
ASS	CCTGAGGGAATTGATGTTGATGAA	CGTGGGCCGTATTGACATCGTG	CCGGTGGCATCAGTTGGCTCATA	383
CAR	GCTCTTCTTGCTCCTTACTC	CGTCATGGCCAGTAGGGAAG	CATGCCAGCATCTAAGCACT	232
CPS	CAGCTGTCCTCCGAATCAC	GAAGGGGCCCGAGAAGTAGAA	CTCAACCGGGGCCAGGAAAAC	445
CYP1A2	TCAGGTCGACTTTCACGCCC	GGAGGCCTTCATCCTGGAGA	TCTCCCACTTGGCCAGGACT	299
CYP2B6	GTTGGCGGTAATGGACTGGAAGA	CCCGCCCTCTGCCCCTTTTG	TCCACACTCCGCTTTCCCATCC	328
CYP3A4	CCCGTGAGAAGCAGAGGA	AGCTTAGGAGGACTTCTTCAACC	AGCCAAATCTACCTCCTCACACT	313
GS	TTGGCAGAGGGGCGACGAT	GCCTGCTTGTATGCTGGAGTC	GGCGCTACGATTGGCTACAC	420
HNF4A	CACTCCAACCCCGCCCCTC	CCGGGTGTCCATACGCATCCT	CAGGTTGTCAATCTTGGCC	321
MRP2	GACGATGATGGTGAAGACAGGAG	AGCACCGACTATCCAGCATCTC	ATCCGGCCTGTGGGTGTTGTG	278
NTCP	GTAGGTGCCATTTCCCAGAGC	GGCTTTCTGCTGGGTTATGTT	GGGGAAAGAAGAAAAGTGGTC	162
OATP2	ATCATCTCTTGGGCATTCACC	GCCGGACTAACCATGACCTAT	GCTGAGTAATTTCTGTTCTGG	250
OTC	GTCAGAGGCAGCAACTTTAGCAG	GCCGGATGCTAGTGTAACCAA	AGCCGCTTTTTCTTCTCCTCTTC	161
PXR	TCTGGTCCTCGATGGGCAAGTC	GAGAGCGGCATGAAGAAGGAGA	CATGTGGGGCAGCAGGGAGAAG	420
TF	CCAGACCACACTTGCCCGCTATG	GAAGGACCTGCTGTTTAAGG	CTCCATCCAAGCTCATGGC	310

Primers are indicated from 5' → 3'.

CHAPTER 5

Liver progenitor cell line HepaRG differentiated in a bioartificial liver effectively supplies liver support to rats with acute liver failure

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ABSTRACT

Background: A major roadblock to the application of bioartificial livers is the need for a human liver cell line that displays a high and broad level of hepatic functionality. The human bipotent liver progenitor cell line HepaRG is a promising candidate in this respect, for its potential to differentiate into hepatocytes and bile duct cells. Metabolism and synthesis of HepaRG monolayer cultures is relatively high and their drug metabolism can be enhanced upon treatment with 2% dimethyl sulfoxide (DMSO).

Aim: To assess the potential of the HepaRG cell line as biocomponent for the AMC-BAL.

Methods: HepaRG cells were cultured in the Academic Medical Center bioartificial liver (AMC-BAL) with and without DMSO and assessed for their hepatic functionality *in vitro* and in a rat model of acute liver failure.

Results: HepaRG-AMC-BALs cultured without DMSO eliminated ammonia and lactate, and produced apolipoprotein A-1 at rates comparable to freshly isolated hepatocytes. Cytochrome P450 3A4 transcript levels and activity were high with 88% and 37%, respectively, of the level of hepatocytes. DMSO treatment of HepaRG-AMC-BALs reduced the cell population and the abovementioned functions drastically. Therefore, solely HepaRG-AMC-BALs cultured without DMSO were tested for efficacy in rats with acute liver failure (n = 6). HepaRG-AMC-BAL treatment increased survival time of acute liver failure rats ~50% compared to acellular-BAL treatment. Moreover, HepaRG-AMC-BAL treatment decreased the progression of hepatic encephalopathy, kidney failure, and ammonia accumulation.

Conclusions: These results demonstrate that the HepaRG-AMC-BAL is a promising bioartificial liver for clinical application.

INTRODUCTION

Acute liver failure (ALF) and acute-on-chronic liver failure are severe syndromes with mortality rates as high as 80%.[1, 2] The syndromes present as a severe impairment of liver function with hepatocellular necrosis, leading to hepatic encephalopathy (HE), systemic inflammation, and multi-organ failure. Despite the progress made in supportive care, liver transplantation is often the only cure, increasing the survival rates to over 80%.[3, 4] However, liver transplantation is limited by the scarcity of donor organs.In the US, about 20% of the patients with severe liver failure (MELD score >30) who are on the waiting list for liver transplantation die while waiting for a donor liver.[5]

Bioartificial livers (BALs) have been developed to bridge these patients to liver transplantation or liver regeneration.[6] BALs typically comprise a bioreactor that is loaded with a biocomponent with hepatic functionality that is connected to the patient's circulation. BAL systems, based on animal hepatocytes, are efficacious in animal models of ALF.[7, 8] However, due to xenotransplantation-related risks, there is an urgent need for BAL systems relying on human biocomponents.[9]

This human biocomponent should compensate for the loss of liver function and counteract concomitant pathology, including hepatic encephalopathy (HE), inflammation, and multi-organ failure. Pathophysiologically, reduced conversion of ammonia into urea and amino acids, as well as reduced drug-metabolizing activity, lead to accumulating levels of ammonia and several other neurotoxins (*e.g.* mercaptans and endogenous benzodiazepines), which all play an important role in the progression of HE.[10] In addition, the lack of several other hepatic functions, including lactate elimination and the synthesis of blood proteins, *e.g.* apolipoprotein A-1, are associated with serious complications, such as lactic acidosis and progressive inflammation.[11, 12] Successful BAL therapy should therefore include the adequate replacement of all these functions.

The human hepatoma cell line HepaRG may be a promising candidate for BAL application. HepaRG is a bipotent liver progenitor cell line that differentiates into two distinct cell populations upon reaching confluence, optionally followed by treatment with 2% dimethyl sulfoxide (DMSO): 1) hepatocyte-like cells that self-organize into clusters; and 2) cluster-neighboring cells that express biliary markers.[13, 14] Cultured on monolayer, DMSO treatment leads to unparalleled high drug-metabolizing activity with expression of *cytochrome P450 (CYP) 3A4* confined to the hepatocyte-like clusters.[13-15]

Recently, we showed that HepaRG monolayer cultures exhibited high metabolic and synthetic properties in the absence of DMSO: the levels of galactose and ammonia elimination, apolipoprotein A-1 production, and transcription of *albumin* reached those of freshly isolated.[16] Interestingly, DMSO treatment promoted cell death and thereby reduced cell-mass more than 2-fold. In addition, DSMO treatment repressed hepatic functions originally present in cluster-neighboring regions. Nonetheless, the drug-metabolizing properties were superior in DMSO-

treated cultures, with transcript levels of *CYP2B6* and *CYP3A4* reaching 61% and 69% of the levels in human liver, whereas the expression levels of these genes reached only 10% and 11%, respectively, in the -DMSO cultures. Taken together, HepaRG cells display a high and broad hepatic functionality promising for BAL application, when HepaRG cultures in the absence and presence of DMSO are being combined.

Therefore, the aim of this study was to characterize the hepatic functionality of the HepaRG cell line differentiated in the AMC-BAL, that was developed by our group, both in the presence and absence of DMSO, and to test the efficacy of this HepaRG-AMC-BAL in a rat model of ALF.[17]

METHODS

Monolayer cell culture and cell isolation

HepaRG cells, isolated from a human liver tumor, were cultured in Hyperflasks (Corning, New York, U.S.) in HepaRG medium without DMSO for large scale expansion.[13] Cultures were maintained at 37ºC in a humidified atmosphere (95% air, 5% CO2). Culture medium was refreshed every 3 to 4 days. We passaged the cells every 2 weeks with a split ratio of 1:6 (cell density ± 10^5 cells/cm^2) using a Accutase (Innovative Cell Technologies, San Diego, U.S.), Accumax (Innovative Cell Technologies), and phosphate-buffered saline (Fresenius Kabi GmbH, Bad Homburg vor der Höhe, Germany) mix of 2:1:1 (v/v/v). Prior to loading the AMC-BAL, the isolated cells were centrifuged at 50x g for 5 min and washed 2x with HepaRG medium. Per AMC-BAL, a suspension of 2 mL cell pellet (approximately 750 million cells) in 9 mL HepaRG medium (= pre-BAL suspension) was prepared.

AMC-BAL culture

We used the laboratory-scale version of the third generation AMC-BAL with an internal volume of 9 mL.[17, 18] AMC-BALs were loaded with the pre-BAL suspension as described previously and from then on oxygenated with a mixture of 40% oxygen, 55% nitrogen, and 5% carbon dioxide (v/v/v).[18] The cells were allowed to attach to the AMC-BAL's matrix for 3 hours. Thereafter, AMC-BALs were continuously perfused with 500 mL recirculating HepaRG culture medium supplemented with 1mM N-carbamoyl-*L*-glutamate (Sigma Aldrich, St. Louis, U.S.). This latter compound was added to increase the ammonia to urea conversion rate ~2-fold.[16] Every 3 to 4 days, all culture medium was refreshed. We generated -DMSO BALs by culturing for 14 days on HepaRG culture medium supplemented with 1mM N-carbamoyl-*L*-glutamate without DMSO (n = 5 to 11). The +DMSO BALs were generated by adding a 14-day culture period on HepaRG culture medium supplemented with 1mM N-carbamoyl-*L*-glutamate and with 2% DMSO (n = 5 to 11) (Sigma Aldrich). At termination of the cultures, the BAL content was lyzed to determine DNA content.[19]

Determination of bioactive mass and cell death

To determine bioactive mass and cell death during AMC-BAL culture, we analyzed pre-BAL suspensions and total lysates of BALs for DNA content (n = 3 to 6). In addition aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) activity were determined in pre-BAL suspensions and in the culture medium perfused through the bioreactor as described previously (n = 3 to 6).[19]

Quantitative reverse transcription-polymerase chain reaction

We analyzed gene expression levels of samples of matrix containing cells (T-bags) harvested from -DMSO and +DMSO BALs as described.[19, 20] Transcript levels were normalized for 18S ribosomal RNA and expressed as a percentage of the mean of two human liver samples. These liver samples derived from two female patients, aged 40 and 41 years, with liver adenoma and no elevated liver damage, after obtaining written informed consent. The patients were not on medication and had no history of drug/alcohol abuse. The procedure was in accordance with the ethical standards of the institutional committee on human experimentation (protocol number 03/024) and the Helsinki Declaration of 1975.

BAL function tests

Bioreactors were first flushed with 30 mL test medium, followed by a 24-hour period of recirculation with 100 mL of test medium (HepaRG culture medium supplemented with 1mM N-carbamoyl-*L*-glutamate, 125 μM testosterone (Sigma Aldrich), 1.5 mM $^{15}NH_4Cl$ (Sigma Aldrich), 2 mM *L*-lactate (Sigma Aldrich), and 2.75 mM *D*-galactose (Sigma Aldrich)). 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, apolipoprotein A-1, and lactate as described.[16, 19, 21, 22] Function parameter rates were determined by calculating the changes in concentration in medium per hour per BAL and per mg DNA harvested from the BALs.

Histology

Complete transverse 8 μm sections of formaline-fixed and paraplast-embedded BALs were stained with hematoxylin (VWR, Amsterdam, The Netherlands) and azofloxine (Sigma Aldrich) as described.[20]

Rat model of complete liver ischemia (CLI)

All procedures were conducted in accordance with the institutional guidelines of the Animal Ethical Committee of the AMC (protocol number 101190). We used a rat model of CLI to induce ALF principally as described.[7] Briefly, male Wistar rats (325 to 350 g; Harlan, Horst, The Netherlands) were treated with 50 mg of the antibiotic rifaximin (Sigma Aldrich) per kg bodyweight, suspended in water, *per gavage* once a day starting from day 5 pre-CLI to standardize

intestinal flora. At day 3 pre-CLI the rats were given an end-to-side portocaval shunt and the animals were allowed to recover for 3 days to prevent bleeding from the shunt anastomosis. For CLI, the hepatic artery was ligated under isoflurane anesthesia and the rats received 8 to 16 IU per 100 g bodyweight of the low molecular weight heparin dalteparine (Pfizer, New York, U.S.) intravenously (t = 0). Subsequently, the rats were allowed to wake up and move around freely. Blood glucose levels were maintained between 5 and 10 mM in all rats. In every experiment, a second rat was used as a blood donor. To this end, blood was drawn intracardially under isoflurane anesthesia and mixed with a citrate-phosphate-dextrose solution (Sanquin, Amsterdam, The Netherlands) in a 7:1 (v/v) blood to solution proportion.

The extracorporeal BAL system

We used a modified version of the extracorporeal BAL system (Fig. 1) used by Flendrig *et al.*[7] Modifications included: 1) incorporating two, instead of one, plasmapheresis modules with an effective length of 105 mm, an internal diameter of 8 mm, 80 Plasmaphan capillaries with 0.47 μm max. pore size, and a total membrane surface of 90 cm^2 (Membrana GmbH, Wuppertal, Germany); 2) priming of the plasma and BAL circuits of the extracorporeal BAL system with plasma expander Elohaes (Fresenius Kabi GmbH) without any supplements; 3) priming of the blood circuit with healthy rat citrate-phosphate-dextrose-blood; 4) supplementing the rat with a 20% dextrose infusion and a saline infusion to a total of 2.0 mL/h to prevent hypoglycemia and dehydration; 5) no continuous dalteparine infusion was given; and 6) placing the extracorporeal BAL system in a temperature-controlled cabinet at 37 ^oC.

Animal study design

Rats were treated with an acellular AMC-BAL (n = 9) (control group) or with a -DMSO HepaRG-AMC-BAL (n = 8) (experimental group). Exclusion criteria were: incomplete recovery from anesthesia, > 6 mL intraperitoneal bleeding post mortem, hemoglobin levels < 9.0 g/dL, pulmonary embolism or stroke, air embolism, and/or incomplete CLI as determined at autopsy. All these exclusion criteria were chosen to secure reproducible progression of ALF until death, without additional confounding factors, *e.g.* >50% blood loss. In the control group, the first 3 rats were excluded because of excessive (> 9 mL) intraperitoneal bleeding. In the experimental group, 3 rats had to be excluded because of pulmonary embolism (thrombus formation in the tubings), air embolism (empty bubble catcher), and incomplete recovery after anesthesia. Notably, these events were unrelated to BAL treatment or the learning curve of the model. At t = 30 minutes, the rat was connected to the extracorporeal BAL system including the AMC-BAL. Blood samples of 0.1-0.5 mL were taken hourly and were replaced with healthy rat citrate-phosphate-dextrose-blood to avoid excessive blood loss. HepaRG-AMC-BALs were refreshed at t = 6 hours to prevent deterioration of BAL function due to ALF plasma toxicity. To prevent dilution by this procedure, the new BAL was filled with plasma harvested from the

preceding BAL. The rat was disconnected from the extracorporeal BAL system after death and autopsy was performed. CLI was considered complete if the liver remained uncolored upon post-mortem injection of a methylene blue solution into the carotid artery, while the caval vein was clamped both inferior and superior to the liver. We did not use histopathology to validate CLI, as this method could miss small remnants of vascularized liver tissue (e.g. by anatomic variation)

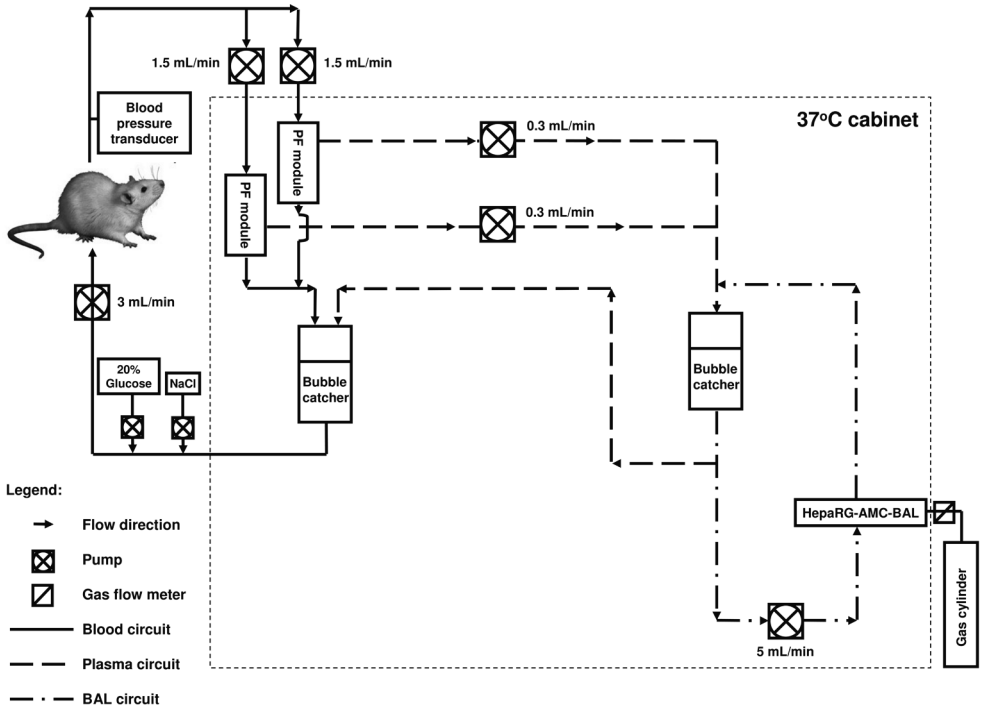

Figure 1. A schematic presentation of the extracorporeal bioartificial liver circuit.

Animal study endpoints

The primary endpoint in this study was survival time, and secondary endpoints were the clinical HE grading score (Table 1), the blood ammonia and the plasma creatinine concentrations. Furthermore, plasma alanine aminotransferase and AST levels, hemoglobin levels, and blood glucose levels were analyzed as described.[7]

Table 1. Clinical grading score of hepatic encephalopathy.

Score	Description
0	Normal behavior
1	Mild lethargy
2	Poor posture control; decreased motor activity; poor posture control
3	No spontaneous righting reflex; severe ataxia; diminished response to pain
4	No righting reflex on pain stimulus
5	Deep coma; no reaction on pain stimulus
6	Death

Statistical analysis

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

RESULTS

Proliferation, cell death, and morphology of HepaRG cells cultured in the AMC-BAL

The total DNA content at the time of loading (pre-BAL) did not significantly differ from the total DNA content of the HepaRG-AMC-BAL after 14 days of culturing without DMSO (-DMSO group) (Fig. 2A). However, in HepaRG-AMC-BALs cultured for an additional 14 days in the presence of 2% DMSO (+DMSO group), the total DNA content decreased to 25% compared to the pre-BAL and the -DMSO groups. The total AST and LDH content per BAL were 134 ± 68 and 312 ± 100 U, respectively. In the -DMSO BALs, AST and LDH leakage were limited, with respectively 0.16 and 0.30 U per BAL per hour, representing a leakage of 2.8% and 2.3%, respectively, of their total content per day (Fig. 2B, C). In the +DMSO BALs, AST and LDH leakage per BAL were comparable to the -DMSO BALs. At a cellular level however, this implied a 3- to 4-fold increase in cell damage in the +DMSO BALs compared to the -DMSO BALs.

The matrix of different cross sections of the -DMSO BALs was completely filled with cells that adhered to the polyester fibers (Fig. 3A, C). Notably, few gas capillaries were present, as they easily detach from the slides during the staining procedure. In contrast to the -DMSO BALs, the matrix of the +DMSO BALs contained many large acellular areas. A closer inspection of these acellular areas revealed the presence a web-like network of fibers typical for extracellular matrix (Fig. 3B, D).

These observations support the hypothesis that DMSO induced cell death in HepaRG-AMC-BALs. In addition, the functionality of -DMSO HepaRG-AMC-BAL remained stable until at least 28 days, excluding the possibility that prolonged culture itself (and not DMSO) could be responsible for the observed cell death (data not shown).

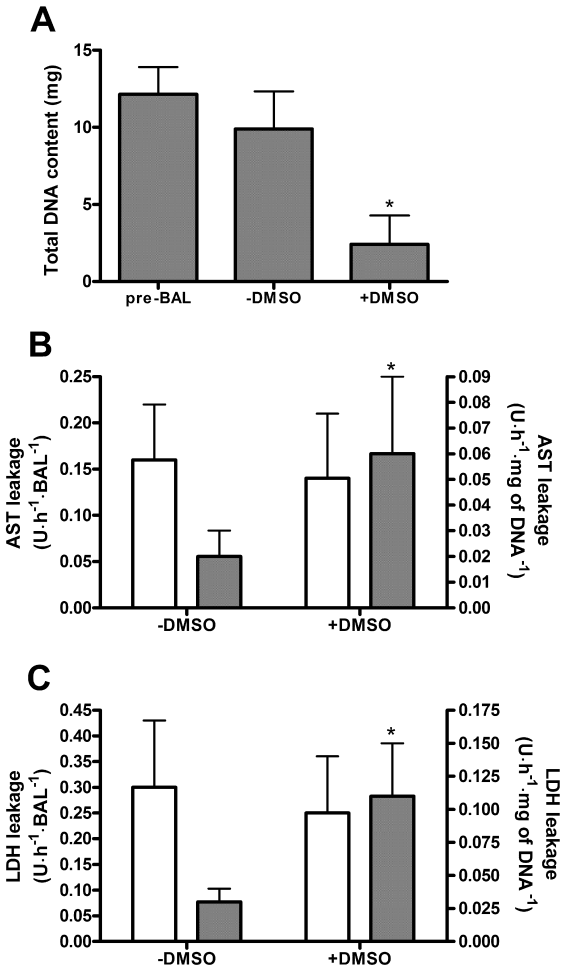

Figure 2. Proliferation and cell death in -DMSO and +DMSO HepaRG-AMC-BALs. Proliferation and cell death in the BALs was measured by their total DNA content (A), and leakage of AST (B) and LDH (C). AST and LDH leakage are expressed 'per BAL' (white bars; read along the left y-axis) and 'per mg of DNA' (grey bars; read along the right y-axis). Values are expressed as means ± standard deviations (n = 3 to 6). Significance: * $p < 0.05$ *versus* pre-BAL suspensions (A) or -DMSO BALs (B, C).

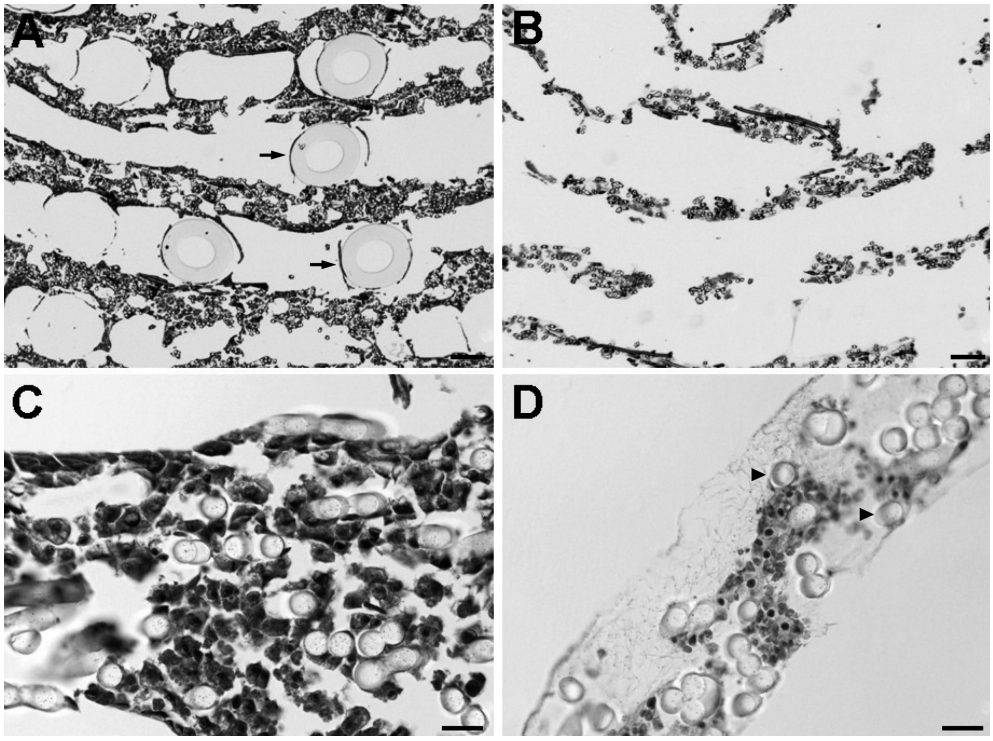

Figure 3. HA stainings of cross sections of -DMSO (A, C) and +DMSO HepaRG-AMC-BALs (B, D). Full transverse sections (A, B) show the spirally wound matrix layers with the gas capillaries (arrows) positioned in between. Details of the matrix (C, D) show the polyester matrix fibers (arrowheads) with HepaRG cells, and the web-shaped extracellular matrix in acellular areas in the matrix (D). Bars: 200 μm (A, B) and 20 μm (C, D). *For color figure, see page 218.*

In vitro functionality of HepaRG-AMC-BALs

Transcript levels of *CYP3A4* and of transcription regulators *hepatic nuclear factor 4α (nuclear receptor subfamily 2, group A, member 1),* and *pregnane X receptor (nuclear receptor subfamily 1, group I, member 2)* in the -DMSO BALs were comparable to human liver (Table 2), and only *hepatic nuclear factor 4α* transcript levels further increased 1.5-fold upon addition of DMSO. Interestingly, *CYP3A4* transcript levels had reached 88% of human liver after 14 days culture without DMSO. Notably, *CYP3A4* transcript levels reached only 10% of human liver in the -DMSO monolayer cultures, and 69% in the +DMSO cultures.[16] The *CYP3A4* transcript levels increased 1.6-fold to 143% of human liver in +DMSO BALs. In contrast to *CYP3A4*, transcript levels of the urea cycle genes *carbamoylphosphate synthetase* and *arginase 1* were only 29% and 16%, respectively, of human liver in -DMSO BALs, and decreased both dramatically to only 1%, upon addition of DMSO. *Glutamine synthetase* transcript levels were 405% of human liver in -DMSO BALs, and also decreased around two-fold upon addition of DMSO.

Table 2. Transcript levels of -DMSO and +DMSO HepaRG-AMC-BALs.

Gene	-DMSO	+DMSO	Fold change	*p* value
Hepatocyte nuclear factor 4α	77 ± 31	116 ± 8	1.5x ↑	0.048
Pregnane X receptor	103 ± 28	181 ± 98	1.8x ↑	0.066
Cytochrome P450 3A4	88 ± 42	143 ± 18	1.6x ↑	0.076
Carbamoyl phosphate synthetase	29 ± 7	1 ± 0	29x ↓	0.000
Arginase 1	16 ± 11	1 ± 1	16x ↓	0.031
Glutamine synthetase	405 ± 202	224 ± 91	1.8x ↓	0.014

Transcript levels are indicated as % of mean mRNA levels of two human liver samples and normalized for 18S ribosomal RNA. The change in transcript levels of +DMSO BALs relative to the -DMSO BALs are indicated with ↑ for upregulation and ↓ for downregulation. Abbreviations: DMSO, dimethyl sulfoxide; AMC-BAL, Academic Medical Center-bioartificial liver. Values are given as means ± standard deviations (n = 5 to 6). *P* values refer to -DMSO *versus* +DMSO BALs.

Overall hepatic functionality in -DMSO BALs was high (Fig. 4), with ammonia elimination (Fig. 4A), 6β-hydroxytestosterone production, a marker for CYP3A4 activity (Fig. 4D), apolipoprotein A-1 production (Fig. 4E), and lactate consumption rates (Fig. 4F) being between 37% and 148% of a porcine hepatocyte BAL (historical control) or freshly isolated human hepatocytes cultured in monolayer.[16, 18] Urea production was still limited with 23% of a porcine hepatocyte BAL. Moreover, analysis of the mass-enriched urea fraction revealed that 27% of the newly produced urea originated from exogenously added ammonia (Fig. 4B, C).

DMSO addition dramatically decreased the hepatic functionality per BAL for all hepatic functions measured. DMSO also reduced some hepatic functions at the cellular level, *i.e.* a 4-fold decreased conversion of ammonia into urea (Fig. 4C) and a switch from lactate consumption to lactate production (Fig. 4F). Therefore, we concluded that -DMSO HepaRG-AMC-BALs outperform +DMSO HepaRG-AMC-BALs in *in vitro* functionality and viability, and therefore only these were tested in ALF rats.

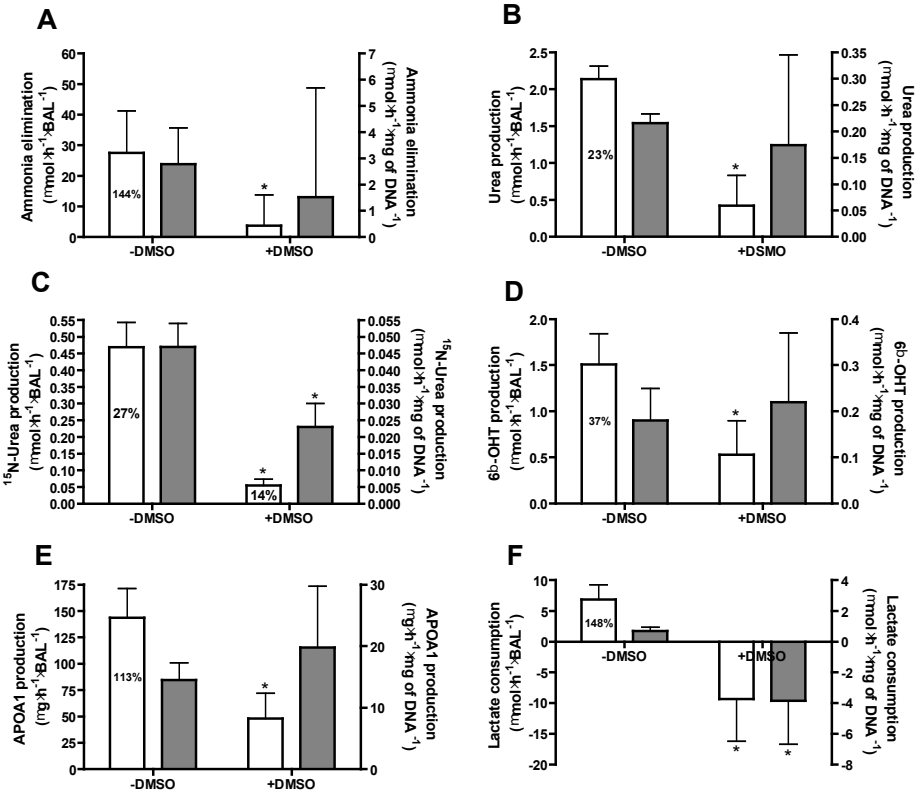

Figure 4. Hepatic functions of the -DMSO and +DMSO HepaRG-AMC-BALs. Ammonia elimination (A), total urea production (B), ^{15}N-urea production (C), 6β-hydroxytestosterone production (D), apolipoprotein A-1 production (E), and lactate consumption (F) of -DMSO and +DMSO HepaRG-AMC-BALs expressed 'per BAL' (white bars; read along the left y-axis) and 'per mg of DNA' (grey bars; read along the right y-axis). Percentages depicted in the bars represent the percentage of this function of freshly isolated porcine hepatocytes cultured in the AMC-BAL (A, B, F) and corrected for the amount of loaded cell pellet, or of freshly isolated human hepatocytes cultured on monolayer (D, E), or the percentage mass-enriched urea of the total amount of produced urea in 24 hours (C). Values are expressed as means ± standard deviations (n = 5 to 11). Significance: * $p < 0.05$ *versus* -DMSO group.

Efficacy of the HepaRG-AMC-BAL in rats with ALF

Two groups of rats underwent CLI: the experimental group, treated with a -DMSO HepaRG-AMC-BAL and the control group, treated with an acellular AMC-BAL. Within 45 min after induction of ALF, all animals had recovered from anesthesia. All rats developed ALF as measured by an increase in plasma alanine aminotransferase and AST levels to >15000 U/L and >20000 U/L, respectively, with no differences between both groups. Post-mortem, CLI was confirmed in all rats using the methylene blue test.

The average survival time in the experimental group was 47% longer than in the control group (14.3 ± 1.9 *versus* 9.7 ± 1.0 hours; $P = 0.001$; n = 5 and 6, respectively) (Fig. 5A). We observed a

gradual progression of HE in all rats (Fig. 5B). However, progression was significantly slower in the experimental group compared to the control group as measured by the time point at which the rats reached the easily observable HE score of 4 for the first time (4.7 ± 1.2 *versus* 9.2 ± 1.6 hours, respectively; $p = 0.005$; n = 6) (Fig. 5C). In all rats blood ammonia levels gradually increased to millimolar levels, but again, slower in the experimental group than in the control group, resulting in a 25% lower average blood ammonia concentration at t=8 hours (Fig. 5D). Plasma creatinine levels were measured to assess renal dysfunction that develops secondary to ALF as part of multi-organ failure. In the experimental group, plasma creatinine levels increased slower, resulting in 57% lower concentration at t = 8 hours (Fig. 5E).

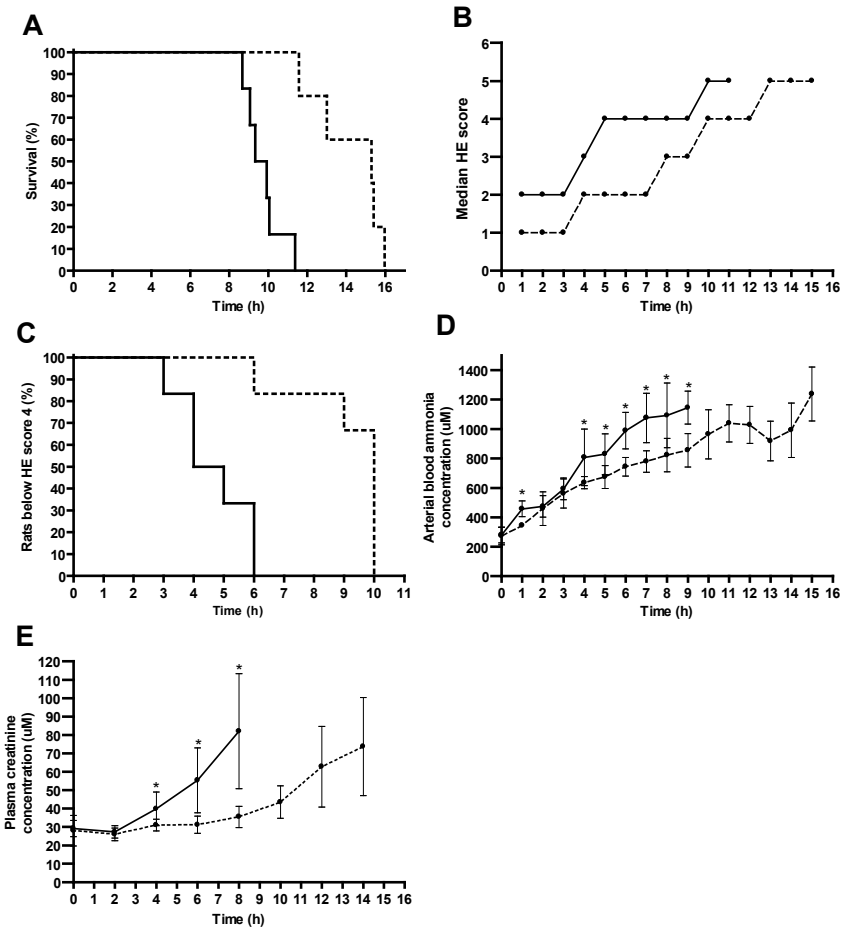

Figure 5. Efficacy of HepaRG-AMC-BAL treatment of rats with ALF. Efficacy was demonstrated by a significantly increased survival time ($p = 0.001$) (A), the median clinical grading score for HE (B), increased time to reach clinical HE score 4 ($p = 0.005$) (C), lower blood ammonia levels (D), and lower plasma creatinine levels (E). Continuous lines indicate the control group and dotted lines indicate the experimental group. Values are expressed as median scores (B), or means ± standard deviations (D, E) (n = 5 to 6). Significance: * $p < 0.05$ *versus* control group.

DISCUSSION

In this study, we demonstrated that the HepaRG-AMC-BAL displays a broad and a high level of hepatic functionality *in vitro*, as measured by the elimination of ammonia, production of urea, production of 6β-hydroxytestosterone, consumption of lactate, and synthesis of apolipoprotein A-1. In addition, we demonstrated that the HepaRG-AMC-BAL. prolongs the life of ALF rats substantially with ~50%.

BAL culture increased hepatic differentiation of liver progenitor cell line HepaRG, particularly its detoxification functionality. CYP3A4 mRNA levels reached 88% of human liver in the -DMSO BALs, whereas this level was only 10% in -DMSO monolayer cultures and ~70% in the presence of DMSO.[16] Hence, DMSO addition becomes superfluous for the upregulation of detoxification functionality of HepaRG cells cultured in the AMC-BAL, as opposed to HepaRG cells cultured in monolayer. This is possibly related to the increased amount of cell-cell contacts that result from the 3D culture environment of the AMC-BAL. Interestingly, a high amount of cell-cell contacts is a prerequisite for high *CYP3A4* expression and activity in freshly isolated human hepatocytes.[23]

In our study, DMSO addition to the culture medium had paramount negative effects on the functionality of HepaRG-AMC-BALs as a whole, probably primarily due to massive (~75%) cell death. On the cellular level, the effects of DMSO were more ambiguous; on the one hand additional negative effects unexplained by cell death, *e.g.* lactate consumption switching into production and additional >2-fold decreased levels of CPS and ARG1 mRNA and of ^{15}N-urea production, with, on the other hand increased HNF4α mRNA levels and a trend towards increased PXR and CYP 3A4 mRNA levels. The mechanism behind the differential effects of DMSO on cell functions is unknown, but might be related to its pleiotropic effect.[14, 24, 25] Proposedly, DMSO exerts its differentiating actions by inducing cessation of the cell cycle and hyperacetylation of histones. In addition, DMSO is an inducer of several phase I, II and III drug metabolizing enzymes in hepatocytes including CYP3A4.[26] On the other hand, DMSO represses various other hepatic functions (*e.g.* expression urea cycle genes) of cluster-neighbouring cells in HepaRG monolayer cultures, perhaps due to differential susceptibility of HepaRG cluster cells (with high *CYP3A4* expression) *versus* cluster-neighbouring cells to DMSO toxicity.[16, 27]

In vitro characterization of HepaRG-AMC-BALs demonstrated that this cell line displays a level of high hepatic functionality.[28] Nonetheless, further improvements of the HepaRG-AMC-BAL can be envisaged. For example, the rate of ammonia elimination was over 50-fold higher than the rate of ^{15}N-urea production, despite the addition of N-carbamoyl-*L*-glutamate to the culture medium. The metabolic fates of ammonia in the liver comprise either conversion into urea or fixation into the amino acids (glutamine and/or glutamate). As the expression of *GS* was over 400% the level of human liver, it is likely that the majority of eliminated ammonia was converted into glutamine. Glutamine in high concentrations, compromises astrocyte function in

the brain and thereby contributes to the progression of HE.[29] For this reason, urea production is the preferred route for ammonia elimination. The limited conversion of ammonia into urea in the HepaRG-AMC-BAL is probably at least partly related to the low expression levels of the urea cycle enzymes *carbamoyl phosphate synthetase*, *arginase 1*, and *ornithine transcarbamoylase*.[16] Therefore, overexpressing these genes in HepaRG cells would be an interesting strategy to increase urea production.

It is difficult to speculate how an increase of ~50% in survival time in our rat model of ALF would translate to a clinical setting of patients with ALF. However, it is important to note that, unlike in most clinical cases of ALF, the rats have no remnant liver function in our model and the HepaRG-AMC-BALs contained the equivalent of about 15% - 20% of the normal total hepatocyte mass of a rat liver. This amount is too low to provide stand-alone, stable, and adequate liver support. In addition our model leads to instant and complete necrosis of the liver, eliciting massive inflammation. Notably however, our study was not devised to predict the duration of liver supporting capacity in a clinical setting, but to demonstrate the proof of principle that HepaRG-AMC-BAL therapy is efficacious in rats with ALF. To put the results of this study in perspective to BALs that use freshly isolated hepatocytes (the golden standard), the AMC-BAL using freshly isolated porcine hepatocytes as biocomponent is capable of increasing the lifespan of rats with ALF ~2-fold using the same model of CLI.[7] However, this porcine hepatocyte BAL contained the double amount of bioactive mass compared to the HepaRG-AMC-BAL in this study, rendering comparison difficult. Notably, this difference in loading mass is caused by the ability of porcine hepatocytes to attach to the oxygen capillaries, while HepaRG cell solely attach to fibers of the matrix.

In conclusion, we have devised a human cell-based BAL using the liver progenitor cell line HepaRG as biocomponent: the HepaRG-AMC-BAL has a high and broad level of hepatic functionality and has proven efficacy in a rat model of ALF. These results encourage us to test the efficacy of the HepaRG-AMC-BAL in a clinical trial in patients with ALF or acute-on-chronic liver failure.

ACKNOWLEDGMENTS

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CHAPTER 6

Increased hepatic functionality of the human hepatoma cell line HepaRG cultured in the AMC bioreactor

Submitted

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ABSTRACT

Background: The clinical application of a bioartificial liver (BAL) depends on the availability of a human cell source with high hepatic functionality, such as the human hepatoma cell line HepaRG. This cell line has demonstrated high hepatic functionality, but the effect of BAL culture on its functionality in time is not known.

Aim: To study the characteristics of the HepaRG-AMC-BAL over time, and compare the functionality of the HepaRG-AMC-BAL with monolayer cultures of HepaRG cells.

Methods & Results: Histological analysis of 14-day-old BALs demonstrated functional heterogeneity similar to that of monolayer cultures. Hepatic functionality of the HepaRG-AMC-BALs increased during 2-3 weeks of culture. The majority of the measured protein-normalized hepatic functions were already higher in day 14 BAL cultures compared to monolayer cultures, including ammonia elimination (3.2-fold), urea production (1.5-fold), conversion of ^{15}N-ammonia into ^{15}N-urea (1.4-fold), and cytochrome P450 3A4 activity (7.9-fold). Moreover, lactate production in monolayer cultures switched into lactate consumption in the BAL cultures, a hallmark of primary hepatocytes, and protein-normalized cell damage was 4-fold lower in day 14 BAL cultures compared to monolayer cultures. Transcript levels of *cytochrome P450* genes and of regulatory genes hepatic nuclear factor 4α and pregnane X receptor increased in time in BAL cultures and reached higher levels than in monolayer cultures. Lastly, metabolism of amino acids, particularly the alanine and ornithine production of HepaRG-AMC-BALs more resembled that of primary hepatocytes than monolayer HepaRG cultures.

Conclusions: We conclude therefore that BAL culture of HepaRG cells increases its hepatic functionality, both over time, and compared to monolayer, and this is associated with a reduction in cell damage, upregulation of both regulatory and structural hepatic genes, and changes in amino-acid metabolism.

INTRODUCTION

Bioartificial livers (BALs) have been developed to bridge patients with acute and acute-on-chronic liver failure to liver transplantation or liver regeneration.[1] BALs typically comprise a bioreactor loaded with a biocomponent (*e.g* hepatocytes) and are able to replace the failing liver when connected to the patient's circulation.

The AMC-BAL that was developed in our laboratory is based on a bioreactor with an internal oxygenator and a spirally wound, non-woven polyester matrix, yielding a three-dimensional culture environment that provides direct cell-plasma contact and optimal oxygenation of the biocomponent.[2]

BAL therapy should compensate for the loss of liver function that gives rise to hepatic encephalopathy, inflammation, and multi-organ failure. Functionally, this entails, among others, the elimination of ammonia, drug-metabolizing activity (*e.g.* cytochrome p450 (CYP) activity), lactate elimination, and the synthesis of blood proteins, such as clotting factors and apolipoprotein A-1.[3-5]

The human hepatoma cell line HepaRG is a promising candidate in this respect. HepaRG is a bipotent liver progenitor cell line that, in monolayer, differentiates into two distinct cell populations in a 28-day period. It differentiates upon reaching confluence, optionally followed by treatment with 2% dimethyl sulfoxide (DMSO) into: 1) hepatocyte-like cells that self-organize into clusters; and 2) cluster-neighboring cells that express biliary duct cell markers.[6, 7]

We confirmed that DMSO treatment increased the expression of *CYP* genes 4- to 20-fold in HepaRG monolayers, leading to *e.g.* a final CYP3A4 activity of 19% of primary human hepatocytes.[8] However in the absence of DMSO a high level of ureagenesis, ammonia elimination, and hepatic protein synthesis was reached, partly due to a higher cell mass.[8] Moreover, when cultured in the laboratory model of the AMC-BAL *without* DMSO for 14 days, HepaRG cells not only demonstrated a high level of ammonia elimination and hepatic protein synthesis, but also a relatively high CYP3A4 activity (6β-hydroxylation of testosterone), reaching 31% of primary human hepatocytes.[9] The high functionality of this HepaRG-AMC-BAL was proven by significantly improving survival time during treatment of rats with acute liver failure.[9]

These results led to the hypothesis that culture of HepaRG cells in the AMC-BAL (in the absence of DMSO) increases their functionality. To investigate this hypothesis, we cultured HepaRG in laboratory scale AMC-BALs for 21 days and assessed hepatic functions at different time points. Subsequently, we compared the state of differentiation of 14 day-old HepaRG-AMC-BALs and HepaRG monolayers, both cultured in absence of DMSO, on basis of their protein-normalized functionality. Finally, we investigated the underlying factors for the upregulated hepatic functionality in the BAL related to cell damage, the expression of both structural and regulatory genes, and amino acid metabolism, the last playing an important role in the regulation of metabolic energy and nitrogen metabolism.[10, 11]

METHODS

Monolayer cell culture

HepaRG cells were kindly provided by prof. C. Guguen-Guillouzo (INSERM, Rennes, France).[6] HepaRG cells were cultured in 24-well culture plates (Corning, New York, U.S.) on HepaRG culture medium without DMSO but supplemented with 1 mM N-carbamoyl-*L*-glutamate (Sigma Aldrich, St. Louis, U.S.) to increase urea production.[8] Analyses were conducted on monolayer HepaRG cultures of 28 days post-seeding.

AMC-BAL culture

For BAL cultures, the HepaRG cells were large-scale expanded in Hyperflasks (Corning, New York, U.S.) in HepaRG medium without DMSO.[6] Cultures were maintained at 37ºC in a humidified atmosphere (95% air, 5% CO2). Culture medium was refreshed every 3 to 4 days. 14-Day-old Hyperflask cultures were harvested using an Accutase (Innovative Cell Technologies, San Diego, U.S.), Accumax (Innovative Cell Technologies), and phosphate-buffered saline (PBS, Fresenius Kabi GmbH, Bad Homburg vor der Höhe, Germany) mix of 2:1:1 (v/v/v). Prior to loading the AMC-BAL, the isolated cells were centrifuged at 50x g for 5 min and washed twice with HepaRG medium.

A suspension of 2 mL cell pellet of HepaRG cells (~750 million cells) was loaded and cultured in laboratory-scale versions (9 mL) of the third generation AMC-BALs (RanD S.r.l. Medolla, Italy) (HepaRG-AMC-BALs) for maximally 21 days on HepaRG culture medium without DMSO and also supplemented with 1 mM N-carbamoyl-*L*-glutamate as described.[9]

Immunohistochemical analysis

Day 14 BALs were formaline-fixed, paraplast-embedded, and cut in complete transverse 8 μm sections.[12] Sections were stained for glutamine synthetase (GS), carbamoyl phosphate synthetase (CPS) and albumin (ALB) as described.[8] In short, sections were incubated with primary antibodies, followed by incubation with alkaline-phosphate labeled secondary antibodies, and visualized using 5-bromo-4-chloro-3'-indolyphosphate p-toluidine and nitroblue. Per staining, all sections were treated similarly and negative controls did not include the primary antibody incubation.

Determination hepatic functions and protein content

Hepatocyte function tests were performed on 28-day-old monolayer cultures (monolayer, n = 6-20) and HepaRG-AMC-BALs after 7 days (d7 BAL, n = 4-11), 14 days (d14 BAL, n = 4-12), and 21 days of culture (d21 BAL, n = 4-6).

For monolayer function tests, cultures were washed twice with phosphate buffered saline. Subsequently, HepaRG culture medium was replaced by 1 mL of HepaRG test medium (HepaRG

medium with 1 mM N-carbamoyl-*L*-glutamate, 1.5 mM $^{15}NH_4Cl$, 2.27 mM D-galactose, 2 mM L-lactate, 125 μM testosterone, and 2 mM ornithine hydrochloride (all compounds from Sigma Aldrich)). Medium samples were taken at different time points of incubation, maximally after 24 hours. Subsequently, cultures were washed twice with PBS and stored at -20°C for protein determination.

For HepaRG-AMC-BAL function tests, bioreactors were first flushed with 30 mL test medium (see above), followed by a 24-hour period of recirculation with 100 mL of test medium. Samples were taken from the test medium prior to connection and after 0.5, 1, 2, 4 and 24 hours of testing.

Test medium samples of monolayer and d7, d14 and d21 BAL cultures were analyzed for concentrations of ammonia, urea, ^{15}N-urea, 6β-hydroxytestosterone (6β-OHT), apolipoprotein A-1, and lactate as described.[8, 13-15] In addition, amino acids concentrations were determined in monolayer and d14 BAL test medium samples.[8] Notably, concentrations of ammonia, 6β-OHT and lactate (other compounds not tested) did not change after 24 hours of perfusion through an acellular bioreactor under the same culture conditions (data not shown). Function-parameter rates were determined by calculating the changes in concentration in medium per hour as described.[15] Of note, the ammonia elimination rates in the HepaRG-AMC-BAL were calculated over the first two hours of testing in HepaRG-AMC-BALs, during which the elimination rate was not reduced by substrate limitation, as occurring at later time points. Likewise, the increase in 6β-OHT concentration in time was not linear at later time points, probably related to subsequent phase-2 detoxification of 6β-OHT. Therefore, the rate of 6β-OHT production was calculated over the first 30 minutes of testing. All other function rates, including metabolism of the different amino acids, were constant over 24 hours of testing.

To study proliferation of HepaRG cells in the AMC-BAL and normalize function parameters for total protein, we determined the total protein content of monolayer cultures (n = 6-20), of cell suspensions at the time of BAL loading (n = 5) and of d14 BALs (n = 7). We lyzed these cells using 0.2 M NaOH (Merck, Darmstadt, Germany), as described.[15] Subsequently, protein analysis was performed using the Coomassie Brilliant Blue G-250 staining kit (Bio-Rad, Hercules, California, U.S.). We normalized hepatic function parameters of monolayer d28 cultures and d14 BAL cultures for protein content.

Determination of cell leakage

To determine cell leakage in monolayer and AMC-BAL cultures of HepaRG, we determined the aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) activity during function tests of monolayer and d14 BAL cultures over a 24-hour period, as described.[13] To relate the measured activities to their total cell contents, the AST and LDH activity per g of protein in total lysates of monolayer cultures (n = 3) and of pieces of cell-filled-matrix extracted from d14 BALs (T-bags; n = 4) were determined as described.[15]

Quantitative reverse transcription-polymerase chain reaction

For gene expression analyses, we isolated RNA samples from (d28) monolayer cultures (n = 3) and from HepaRG-AMC-BAL matrix samples (T-bags) after 7 (n = 3-4), 14 (n = 3-5), and 21 (n = 3) days of BAL culture, using the RNeasy mini kit (Qiagen, Venlo, The Netherlands).[12] First strand cDNA was generated as described using gene-specific reverse transcription primers.[14] The quantitative reverse transcription polymerase chain reaction was performed as described.[15] Transcript levels were normalized for 18S ribosomal RNA and calculated as the mean levels of two human liver samples that were included in the analysis as described.[16] These liver samples derived from two female patients, aged 40 and 41 years, with liver adenoma and no elevated liver damage, after obtaining written informed consent. The patients were not on medication and had no history of drug/alcohol abuse. The procedure was in accordance with the ethical standards of the institutional committee on human experimentation (protocol number 03/024) and the Helsinki Declaration of 1975. Primer sequences and amplicon sizes are depicted in Table 1.

Statistical analysis

We compared differences between two experimental groups using unpaired Student's t-tests. Differences between three or more experimental groups were compared using one-way analysis of variance (ANOVA) with Tukey post-hoc analysis. 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 $p < 0.05$.

Table 1. Primer sequences used in the RT-PCR analyses with resulting amplicon sizes.

Gene	RT primer	Sense primer	Antisense primer	Amplicon size (bp)
18S rRNA	CGAACCTCCGACTTTCGTTT	TTCGGAACTGAGGCCATGAT	CGAACCTCCGACTTTCGTTCT	151
ALB	ACTTCCAGAGCTGAAAAGCATGGTC	TGAGCAGCTTGGAGAGTACA	GTTCAGGACCACGGATAGAT	189
ARG1	TGTGATTACCCTCCCGAGCAAGTC	TTGGCAAGGTGATGGAAGAAACA	CCTCCCGAGCAAGTCCGAAACAA	305
CPS	CAGCTGTCCTCCGAATCAC	GAAGGGGCCCGAGAAGTAGAA	CTCAACCGGGGCCAGGAAAAC	445
CYP1A2	TCAGGTCGACTTTCACGCCC	GGAGGCCTTCATCCTGGAGA	TCTCCCACTTGGCCAGGACT	299
CYP2B6	GTTGGCGGTAATGGACTGGAAGA	CCCGCCCTCTGCCCCTTTTG	TCCACACTCCGCTTTCCCATCC	328
CYP3A4	CCCGTGAGAAGCAGAGGA	AGCTTAGGAGGACTTCTTCAACC	AGCCAAATCTACCTCCTCACACT	313
CYP3A5	GGTACCATCTCTTGAATCCACC	TGACCCAAAGTACTGGACAG	TGAAGAAGTCCTTGCGTGTC	240
CYP3A7	AGCCAAATCTACTTCCCCAGCAC	ATTACGCTTTGGAGGACTTCTTCT	CGTCTTCATTTCAGGGTTCTATTT	182
GS	TTGGCAGAGGGGCGACGAT	GCCTGCTTGTATGCTGGAGTC	GGCGCTACGATTGGCTACAC	420
HNF4A	CACTCCAACCCCGCCCCTC	CCGGGTGTCCATACGCATCCT	CAGGTTGTCAATCTTGGCC	321
PXR	TCTGGTCCTCGATGGGCAAGTC	GAGAGCGGCATGAAGAAGGAGA	CATGTGGGGCAGCAGGGAGAAG	420

Primers are indicated from 5' → 3'.

RESULTS

Heterogeneous distribution of hepatic proteins of HepaRG-AMC-BALs

We stained transverse sections of d14 BALs for the hepatic proteins GS, CPS, and ALB. The HepaRG cells resided in the matrix of the BALs, were all vital, and the three cytosolic proteins were heterogeneously distributed among the cells (Fig. 1A-F), as found previously for -DMSO monolayer cultures.[8] Notably, the distribution of ALB concentrated at the rims of cell-clusters, which possibly reflects an accumulation of this protein at the secretion site.

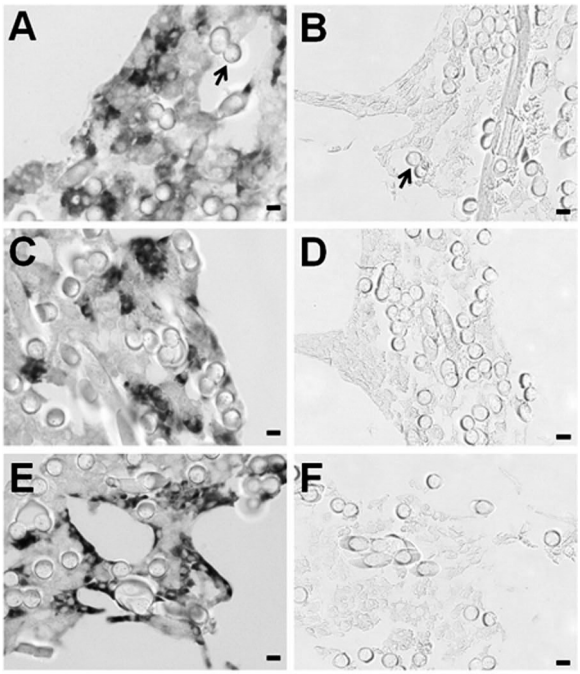

Figure 1. Immunostainings of cross-sections of d14 HepaRG-AMC-BALs. Proteins stained were GS (A) with its negative control (B), CPS (C) with its negative control (D), and ALB (E) with its negative control (F). Fibers of the AMC-BAL's matrix are indicated with arrows. Bars: 10 μm. *For color figure, see page 219.*

Hepatic functionality of HepaRG-AMC-BALs increases with time

We compared various hepatic functions of HepaRG-AMC-BALs during three weeks of culture (Fig. 2). From d7 to d21 the urea production (Fig. 2B), 15N-urea production (Fig. 2C), 6β-OHT production (Fig. 2D), and apolipoprotein A-1 production (Fig. 2E) increased (in most cases gradually) 3.2-, 2.2-, 5.1-, and 2.2-fold, respectively, whereas ammonia (Fig. 2A) and lactate production (Fig. 2F) remained stable.

The increased functionality was (at least for the first 14 days) not due to an increase in proliferation in the AMC-BAL, as the total protein content of the HepaRG-AMC BAL at the time of loading (149 ± 18 mg) was not significantly different from the total protein content after 14 days of BAL culture (163 ± 16 mg) ($p = 0.20$). This is in line with the finding that the total DNA content neither changes during the first 14 days of BAL culture.[9]

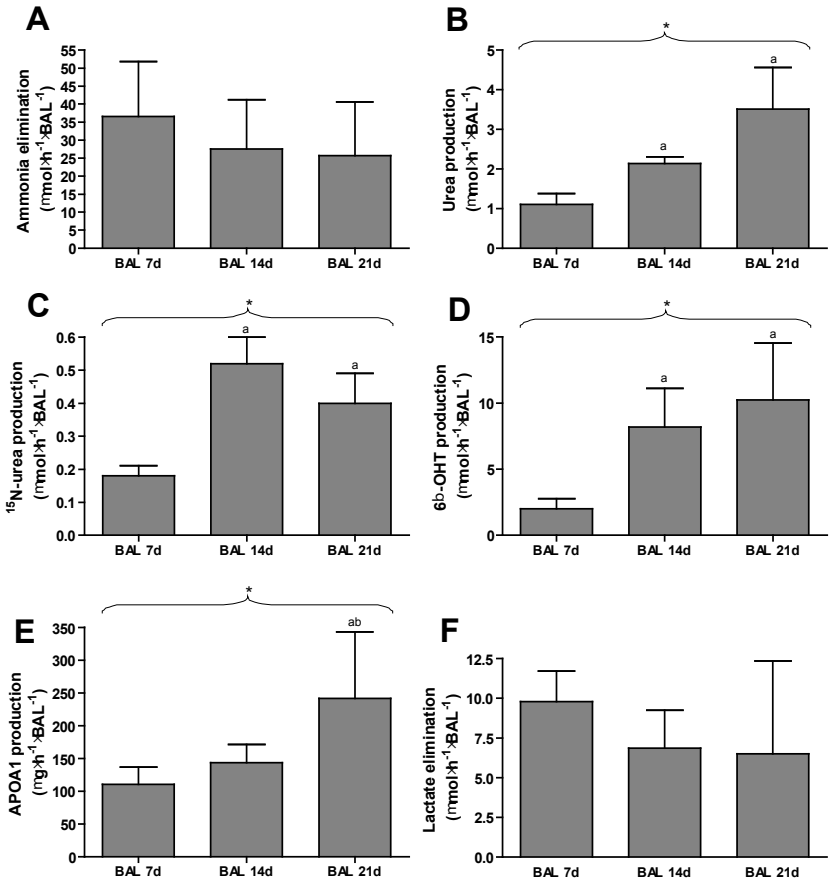

Figure 2. Hepatic functions of d7, d14, and d21 HepaRG-AMC-BALs. Values are expressed as means ± standard deviations. Significance: * above accolade indicates $p < 0.05$ between all groups, $^{a}p < 0.05$ *versus* d7 HepaRG-AMC-BALs, $^{b}p < 0.05$ *versus* d14 HepaRG-AMC-BALs.

HepaRG-AMC-BALs reach higher hepatic functionality than HepaRG monolayer cultures

We compared protein normalized functional parameters of HepaRG-AMC-BAL after 14 days of culture with 28-day-old monolayer HepaRG cultures (Fig. 3A-F). All functions assessed were substantially higher in HepaRG-AMC-BALs than in HepaRG monolayer cultures, except for the

apolipoprotein A-1 production (Fig. 3E), which was equal. The relative increases were 3.2-fold for ammonia elimination, 1.5-fold for urea production, 1.4-fold for conversion of ^{15}N-ammonia into ^{15}N-urea, and 7.9-fold for CYP3A4 activity. Moreover, lactate production (Fig. 3F) in monolayer cultures had switched into lactate consumption in HepaRG-AMC-BALs, a hallmark of BAL cultures of primary hepatocytes.[17]

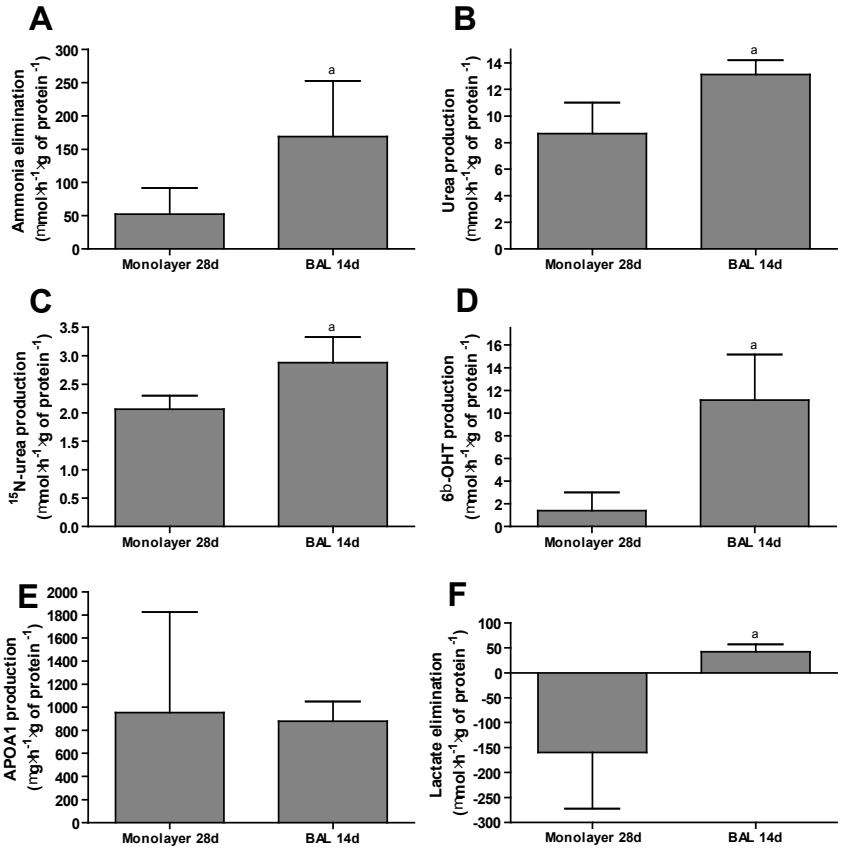

Figure 3. Hepatic functions of 28-day monolayer cultures of HepaRG cells and d14 HepaRG-AMC-BALs. Values are expressed as means ± standard deviations. Significance: $^{a}p < 0.05$ *versus* monolayer cultures.

Cell leakage in HepaRG-AMC-BALs is lower than in monolayer cultures of HepaRG

As one of factors potentially related to the BAL-culture induced increase in hepatic functionality of HepaRG cells, we compared cell damage in monolayer cultures and d14 HepaRG-AMC-BALs. As markers for cell damage, we measured the amounts of AST and LDH leakage per hour, expressed as a fraction of the total amount of AST and LDH content in the cell cultures. Monolayer cultures and HepaRG-AMC-BALs contained 318 ± 28 and 896 ± 442 U AST/g

protein, respectively, and 789 ± 442 and 755 ± 181 U LDH/g protein, respectively. The AST and LDH leakage of the monolayer and d14 BAL cultures corrected for total cellular contents was between 0.1 and 1% per hour, with d14 BAL cultures displaying a 4-fold lower leakage compared to the monolayer cultures (Fig. 4).

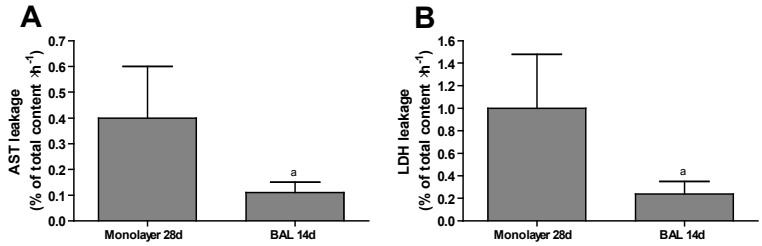

Figure 4. AST leakage (A) and LDH leakage (B) of monolayer cultures of HepaRG cells and d14 HepaRG-AMC-BALs. Cell leakage is expressed as the percentage of the total AST and LDH content of the entire culture per hour ± standard deviations. Significance: $^{a}p < 0.05$ *versus* 28-day monolayer cultures.

Transcript levels of both structural and regulatory hepatic genes increase upon BAL culture of HepaRG cells.

In addition, we investigated whether the observed differences between monolayer and BAL culture, as well as the increase in functionality *during* BAL culture, are regulated on a transcriptional level.

Transcript levels of genes encoding rate-limiting urea cycle enzymes *CPS* (Fig. 5A) and *arginase 1 (ARG1)* (Fig. 5B) were not significantly different between monolayer and BAL cultures, and remained stable during BAL culture, although we observed a trend towards an increase for ARG1 in d21 HepaRG-AMC-BALs ($p = 0.14$ *vs* monolayer). The transcript level of the gene encoding GS, the enzyme catalyzing the conversion of glutamate into glutamine thereby eliminating ammonia, was higher during the whole period of BAL culture compared to monolayer, being the highest in d7 HepaRG-AMC-BALs (Fig. 5C). In general, the transcript levels of the measured *CYP* genes showed a trend towards an increase during BAL culture, with maximal levels being higher than those in monolayer (Fig. 5 D-G). Most importantly, the transcript level of *CYP3A4*, the gene encoding the most dominant CYP in human liver, increased strongly (10.4-fold) upon BAL culture to reach a level at day 21 comparable to human liver (Fig. 5F).

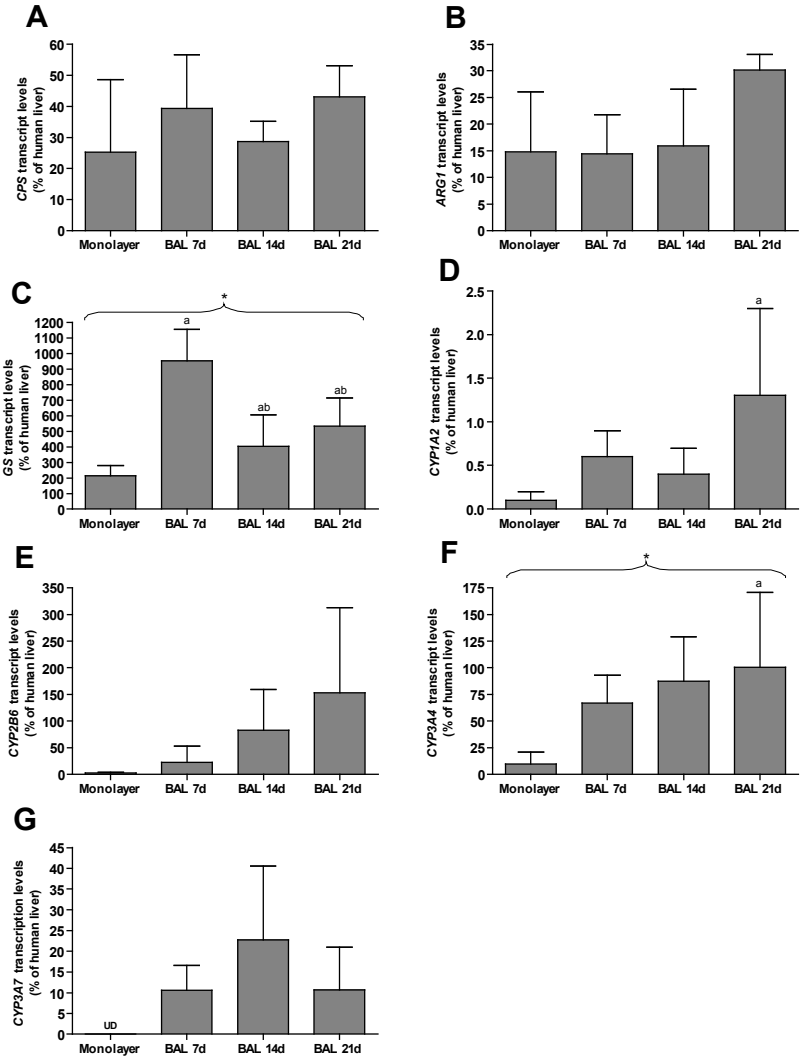

Figure 5. Transcript levels of various structural hepatic genes of 28-day-old monolayer cultures of HepaRG cells and 7-, 21-, and d14 HepaRG-AMC-BALs. Values are expressed as means ± standard deviations. Abbreviations: *ARG1, arginase 1; CPS, carbamoylphosphate synthetase; CYP, cytochrome P450; GS, glutamine synthetase;* UD, undetectable. Significance: * above accolade indicates $p < 0.05$ between all groups, $^{a}p < 0.05$ *versus* monolayer cultures, $^{b}p < 0.05$ *versus* d7 HepaRG-AMC-BALs.

We determined the transcript levels of two hepatic transcription factors: *hepatic nuclear factor 4α (HNF4α; nuclear receptor subfamily 2, group A, member 1)*, an important regulator of morphological and functional liver development (including drug metabolism) and a repressor

of dedifferentiation, and *pregnane X receptor (PXR; nuclear receptor subfamily 1, group I, member 2)*, a master regulator of drug metabolism.[18, 19] Both *HNF4α* and *PXR* transcript levels increased upon BAL culture (Fig. 6A, B) from 50% and 41% the level in human liver to 245% and 142% the level of human liver, respectively, and they may therefore contribute to the observed increase in hepatic functionality, including drug metabolism.[20]

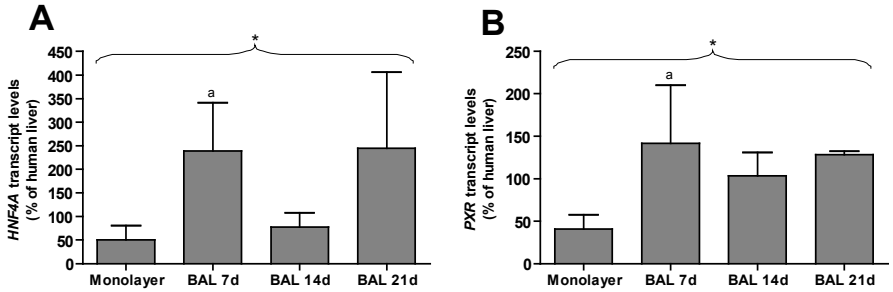

Figure 6. Transcript levels of regulatory hepatic genes of 28-day-old monolayer cultures of HepaRG cells and 7-, 21-, and d14 HepaRG-AMC-BALs. Values are expressed as means ± standard deviations. Abbreviations: *HNF4A, hepatic nuclear factor 4A; PXR, pregnane X receptor.* Significance: * above accolade indicates $p < 0.05$ between all groups, $^{a}p < 0.05$ *versus* monolayer cultures.

Amino-acid metabolism

Total amino-acid consumption was similar in monolayer and d14 HepaRG-AMC-BALs (Table 2). However, the net metabolism of individual amino-acids varied between both cultures. Net glutamate consumption in BAL cultures was more than 2-fold lower than in monolayer cultures, while glutamine production remained constant. In contrast, alanine was *produced* at a net rate of 20.5 ± 4.75 μmol/h in monolayer cultures, whereas it was *consumed* at a net rate of 5.85 ± 2.51 μmol/h in HepaRG-AMC-BALs. This might reflect a BAL-culture induced shift in the transamination reaction catalyzed by alanine transaminase, resulting in the increased production of pyruvate and glutamate (see Discussion section).

Ornithine was produced in BAL cultures, whereas it was consumed in monolayer cultures, and we also observed a trend towards an increase in arginine consumption in BAL cultures compared to monolayer cultures ($p = 0.05$). These findings are in accordance with the observed increase in urea production by arginase 1 upon BAL culture. Lastly, leucine was consumed at a lower rate in BAL cultures compared to monolayer cultures, while the metabolism of the other branched chain amino acids did not differ upon BAL culture.

Table 2. Amino acid metabolism of HepaRG monolayer cultures and HepaRG-AMC-BALs.

Amino acid	HepaRG Monolayer ($\mu mol \cdot h^{-1} \cdot g$ of $protein^{-1}$)	HepaRG-AMC-BAL 14d ($\mu mol \cdot h^{-1} \cdot g$ of $protein^{-1}$)
ALA	20.5 ± 4.75	-5.85 ± 2.51 [a]
ARG	-1.64 ± 0.90	-3.66 ± 1.26
ASN	-1.31 ± 0.86	-0.77 ± 0.44
ASP	N/A	N/A
CIT	-0.15 ± 0.33	-0.24 ± 0.60
GLN	7.15 ± 3.19	9.72 ± 2.90
GLU	-16.14 ± 1.19	-7.13 ± 2.17 [a]
GLY	N/A	N/A
HIS	-1.71 ± 0.91	-1.78 ± 0.73
ILE	-7.88 ± 1.28	-6.84 ± 2.02
LEU	-17.55 ± 1.30	-10.66 ± 2.59 [a]
LYS	-10.34 ± 6.13	-9.25 ± 4.59
MET	-2.06 ± 1.20	-1.68 ± 0.57
ORN	-8.57 ± 7.98	1.69 ± 1.30 [a]
PHE	-2.82 ± 0.61	-1.25 ± 0.46 [a]
SER	-4.30 ± 3.44	-1.18 ± 7.11
TAU	-3.73 ± 2.22	-2.70 ± 1.04
THR	-3.92 ± 2.08	-2.81 ± 1.16
TRP	-3.45 ± 2.27	-2.58 ± 1.33
TYR	-0.12 ± 0.16	-0.08 ± 0.07
VAL	-4.35 ± 1.16	-5.06 ± 1.62
Total	-62.36 ± 57.04	-54.05 ± 22.35

Amino acid production and consumption are indicated as positive and negative values, respectively. Abbreviations: AMC-BAL, Academic Medical Center-bioartificial liver; N/A, not available. Values are given as averages ± standard deviations (n = 3 to 5). [a] $p < 0.05$ *versus* HepaRG monolayer cultures.

DISCUSSION

In this study, we demonstrate that culturing of the human hepatoma cell line HepaRG in the AMC-BAL increases its hepatic functionality, both over time, and compared to monolayer. The increase in functionality was most prominent after 14 and 21 days of BAL culture and was associated with a reduction in cell damage, upregulation of both regulatory and structural hepatic genes, and changes in amino-acid metabolism. In addition, the HepaRG cells maintain their functional heterogeneity upon BAL culture, as demonstrated by histological analysis.

The cause of this BAL-culture induced increase in hepatic functionality is most likely multi-factorial. Firstly, the AMC-BAL provides a three-dimensional culture environment whereas monolayer is two-dimensional. Three-dimensional cultures of hepatocytes more resemble the *in vivo* cellular organization in terms of cell-cell contacts, polarity, morphology, and the composition of the extracellular matrix.[21, 22] In addition, the AMC-BAL has an internal

oxygenator, providing superior oxygenation of the cells compared to monolayer, known to increase functionality.[23-25] Lastly, the AMC-BAL is perfused with culture medium in contrast to the static culture environment of the monolayer cultures, also known to increase functionality.[26]

Gene expression analysis revealed that the difference in hepatic functionality in d14 HepaRG-AMC-BALs *vs* HepaRG monolayer cultures may be partially explained by regulation on a transcriptional level. For instance, the 7.9-fold increase in CYP3A4 activity (6β-OHT production) coincided with a comparable 10.4-fold increase in *CYP3A4* transcript levels, suggesting a transcriptionally regulated increase of CYP3A4. This may well be caused by the observed upregulation of both *PXR* and *HNF4A* expression, both important transcriptional regulators of *CYP3A4*.[20] The increase in ammonia elimination may likewise be regulated on a transcriptional level, as *GS* transcript levels were significantly higher in the d14 BAL cultures compared to the monolayer cultures. Notably, ammonia elimination in HepaRG cells is mainly realized by fixation into amino acids (initially glutamate and glutamine), and not by conversion into urea. Both ^{15}N-urea production and total urea production increased significantly upon BAL culture of HepaRG cells, notwithstanding the lack of a significant increase in the transcription level of CPS and arginase 1, suggesting regulation at a posttranscriptional level, or regulation by other factors or enzymes involved in the urea cycle.

The amino acid metabolism in HepaRG-AMC-BAL cultures more resembled the metabolism of primary hepatocytes than that of HepaRG monolayer cultures.[2] In contrast to monolayer HepaRG cultures, both primary porcine hepatocyte and HepaRG BAL cultures produced alanine and ornithine. However, glutamate metabolism of HepaRG-AMC-BALs (consumption) differs with primary-porcine-hepatocyte-AMC-BALs (production). This difference might be related to the high GS activity in HepaRG-AMC-BALs compared to primary porcine hepatocyte AMC-BALs.[2] Furthermore, we observed a substantial decrease in glutamate consumption upon BAL culture of HepaRG cells and a transition from alanine production into consumption. This suggests that the transamination reaction catalyzed by alanine aminotransferase in which glutamate and pyruvate are converted into alanine and α-ketoglutarate and *visa versa* shifts towards the production of glutamate and pyruvate. This may, in part, explain the discrepancy between a high increase in elimination of ammonia and the limited change in glutamate/glutamine metabolism in HepaRG cells (< 10% of the change in ammonia elimination) of 14d BALs versus monolayers. The higher ^{15}N-urea production in 14d BALs relative to monolayers coincided with a switch from ornithine consumption into production, and a strong trend towards an increase in arginine consumption ($p = 0.05$), the respective product and substrate of the ARG1 reaction in the urea cycle.

In addition, the amino acid, ammonia, and urea data provide interesting insights in the nitrogen metabolism of the HepaRG-AMC-BAL. The rate of ammonia elimination is 168 μmol per hour per gram of protein. The net total amino acid consumption is 54 μmol per hour per gram of protein. This equates to a total influx of at least 222 μmol of nitrogen per hour per

gram of protein – at least, as several amino acids also contain nitrogen atoms in the side chain next to the amine group. However, the rate of total urea production is only 13 μmol per hour per gram of protein, covering the elimination of only 26 μmol of ammonia per hour. The majority of the nitrogen that is taken up by the HepaRG cells must therefore be used for protein synthesis, and as the total protein content in the HepaRG-AMC-BAL does not change during two weeks of culture, this suggests a substantial protein secretion. This would also explain the lack of stoichiometry in the changes in ammonia elimination and urea production by quantitative amino acid analysis in our set-up.[9]

In monolayer cultures, we observed lactate production, whereas BAL cultures of HepaRG cells consumed lactate. Lactate consumption is a liver-specific function and part of the Cory cycle, in which lactate that is produced by anaerobic glycolysis in the muscles and subsequently transported to the liver where it is converted into pyruvate and later glucose. Lactate consumption is also a hallmark of freshly isolated hepatocytes.[17] In contrast, liver cell cultures *produce* lactate under anaerobic conditions or when the cells have dedifferentiated. The latter effect, known as the Warburg effect, is also a feature of tumor cells as a means of rapid cytosolic energy production.[27, 28] Based on the increased hepatic functionality upon culturing the HepaRG cells in the BAL, the shift from lactate production into consumption can probably be attributed to the higher differentiation state of the HepaRG cells rather than to the increased oxygenation in the BAL versus monolayer, however the causal relation between oxygenation, lactate metabolism and hepatic differentiation needs to be further established.

In conclusion, the results of this study contribute to the clinical implementation of a BAL based on the HepaRG cells, as it shows that BAL culture improves its hepatic functionality. Notably, the functionality of a HepaRG-AMC-BAL is higher and more all round than that of a DMSO-treated HepaRG monolayer culture, without the DMSO-associated negative effects as cell damage and repression of particular hepatic functions.[8, 9] The d21 BALs functionally outweigh d14 BALs, raising the question whether further extension of the BAL culture period, *i.e.* 4 weeks and longer, would further increase the hepatic functionality of the HepaRG-AMC-BAL. This also offers high perspectives for BAL therapy, as the d14 HepaRG-AMC-BAL was already effective in the treatment of rats with ALF.[9] Finally, this study further encourages clinical studies with the HepaRG-AMC-BAL to evaluate its safety and efficacy in patients with ALF.

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CHAPTER 7

The effect of rat acute-liver-failure plasma on HepaRG cells

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ABSTRACT

Background: Recently, we have demonstrated high liver functionality, including ammonia eliminating capacity of the human liver cell line HepaRG, rendering it a valuable biocomponent of a bioartificial liver (BAL) to support patients with acute liver failure (ALF). This cell line further gains detoxification properties when cultured with dimethyl sulfoxide (DMSO).

Aim: In this paper we describe whether its functionality will be compromised by toxic effects of ALF plasma, as has been shown for primary hepatocytes.

Methods: We exposed -DMSO and +DMSO HepaRG cultures during 16 hours to healthy- and ALF-rat plasma. The cultures were analyzed for lipid accumulation, cell leakage, apolipoprotein A-1 production, nitrogen metabolism and transcript levels of hepatic genes.

Results: The -DMSO cultures showed increased cell leakage after healthy and ALF plasma exposure in contrast to +DMSO cultures, but otherwise the -DMSO and +DMSO cultures were equally affected by exposure to the plasmas. Exposure to both plasmas caused lipid accumulation and decreased transcript levels of various hepatic genes. ALF plasma decreased urea cycle activity, but increased urea production from arginine by upregulated *arginase 2*. However, total ammonia elimination was not affected by exposure to either plasma, indicating its predominant elimination by fixation into amino acids. In addition apolipoprotein A-1 production remained constant.

Conclusions: HepaRG cells are negatively affected by rat plasma, even of healthy origin. However, their ammonia eliminating capacity is relatively resistant, underlining their suitability for BAL application. DMSO pre-treatment may increase their viability in plasma.

INTRODUCTION

Bioartificial livers (BALs) have been developed to bridge patients suffering from acute liver failure (ALF) to liver transplantation or liver regeneration.[1] BALs rely on bioreactors with liver cells that compensate for the loss of functional liver-cell mass in ALF patients. The required BAL functions are not specified, however conversion of ammonia into urea, drug-metabolizing capacity and synthesis of blood proteins are probably important.[2, 3]

Ideally, BAL systems contain a biocomponent of human origin with sufficient proliferative and functional capacity. In this respect, the HepaRG cell line is a promising biocomponent.[4] The HepaRG cell line is a bipotent progenitor cell line from a human hepatocellular carcinoma that, in monolayer, starts to differentiate at high confluence (at 14 days after seeding) into a culture with clusters of cuboid and granular hepatocyte-like cells, surrounded by flattened cells. A successive culture phase of 14 days in the presence of 2% dimethyl sulfoxide (DMSO) upregulates components of drug-metabolism, like cytochrome P450 (CYP) 3A4.[5]

Our recent investigation on liver specific functions relevant for BAL application, confirmed a unique high hepatic functionality of HepaRG cells with ammonia elimination and apolipoprotein A-1 (ApoA1) production rates similar to those of primary human hepatocytes. The ammonia elimination occurred probably mainly through fixation into amino acids, particularly through Glutamine Synthetase (GS) activity. However we also showed that DMSO treatment elicited undesirable effects; a reduction in cell mass, galactose elimination and transcript levels of hepatic genes including the rate limiting urea cycle enzyme *carbamoyl phosphate synthetase (CPS)* and *GS*.[6]

The abovementioned performance of HepaRG cells was tested in culture medium. However, their suitability as biocomponent for BALs relies on their performance during exposure to ALF plasma, since cytotoxic compounds accumulating in blood during ALF decrease the functionality of liver cells.[7] These toxic compounds in ALF include components of necrotic cells, the activated innate immune system and substrates that are normally metabolized by the liver. Components of ALF plasma with proven cytotoxic effects are ammonia, lactate, bile acids, bacterial lipopolysaccharide and the cytokines IL-6, TNF-α and IL-1β.[7-11]

The cytotoxic effects of ALF plasma on liver cells comprise a disturbance in cell morphology and a reduction in cell viability and function. Importantly, a severe decline in drug-metabolizing and ammonia eliminating activity has been observed in monolayer and in BAL cultures of primary hepatocytes after exposure to ALF plasma.[12-14] Human liver cell lines have been less studied in this respect; however changes in morphology, a reduction in viability, CYP activity and urea production have been reported.[15-19]

Moreover, exposure to healthy plasma may already elicit negative effects in hepatocytes, including lipid droplet formation, a reduction in urea, albumin and glutathione production.[20, 21] In contrast, an increase in CYP activity and stable urea production has also been reported.[16, 17, 22, 23] These controversies may relate to differences in cell types and plasmas used, as well as to differences in exposure times.

For HepaRG cells however, the effects of ALF or healthy plasma have not been reported yet. Therefore, the aim of our study was to assess the response of HepaRG cells to exposure to ALF or healthy plasma in the context of BAL therapy. We hypothesized that a high drug metabolizing and ammonia eliminating capacity may yield relatively high resistance to these plasmas.

Therefore, we exposed -DMSO and +DMSO HepaRG cultures to healthy- and ALF-rat plasma, and compared their characteristics with cultures maintained on culture medium, this way isolating the specific responses to healthy plasma and to ALF components in the plasma. ALF plasma from rats was used, as this was available in high quantities and was expected to contain high levels of cytotoxins, being harvested immediately after death from ALF.

METHODS

Cell culture

HepaRG cells were kindly provided by prof. C. Guguen-Guillouzo (INSERM, Rennes). HepaRG cells were seeded at 10^5 cells/cm^2 in 24-well culture plates (Corning, New York, U.S.) and subsequently cultured in 0.5 mL HepaRG culture medium / well for 28 days as described previously.[4, 6] The HepaRG culture medium was composed of Williams' E medium (Lonza, Basel, Switzerland) with 10% fetal bovine serum (Lonza), 5 μg / mL insulin (Sigma, St. Louis, U.S.), 50 μM hydrocortisone hemisuccinate (Sigma), 2mM glutamine (Lonza), 50 U / mL penicilline and 50 μg / mL streptomycin (penicilline/streptomycine mix, Lonza). The -DMSO and +DMSO cultures differed in the last 14 days of culture by the absence or presence of 2% DMSO (Sigma), respectively.

Plasma exposure experiments

Plasma was derived from 4 healthy rats (combined) and 4 ALF rats (combined). The ALF plasma was collected immediately from rats after death due to complete liver ischemia as previously described.[24] The state of ALF was confirmed by assessment of the biochemical profile of the plasmas (Table 1). To harvest the plasma, blood was withdrawn from the healthy and ALF rats and collected in BD Vacutainer® lithium heparin plasma separator tubes (Becton Dickinson, Plymouth, UK). The plasma was collected after centrifugation for 10 min at 1300g and subsequently supplemented with 2 IU/mL dalteparine (low molecular weight heparin; Pfizer, New York, U.S.) and 50 U/mL penicillin with 50 μg/mL streptomycin (Lonza,).

The -DMSO and +DMSO HepaRG cultures were exposed to the undiluted plasmas for 16h. Control cultures were maintained on culture medium. After plasma exposure, the cultures were washed twice with phosphate-buffered saline (Fresenius Kabi GmbH Bad Homburg vor der Höhe, Germany). Subsequently, either RNA was immediately harvested for reverse transcription-polymerase chain reaction, or the cultures were subjected to a hepatocyte function test or staining for lipid droplets.

Table 1. Biochemical profile of the pooled plasmas (from 4 animals/group) utilized in this study.

Source	Ammonia (μM)	Total bilirubin (μM)	AST (U/L)	LDH (U/L)	Triglycerides (mM)
Healthy rat	20	<1	99	631	1.80
ALF rat	1238	15	>6000	>3000	0.56

Abbreviations: ALF, acute liver failure; AST, Aspartate aminotransferase; LDH, lactate dehydrogenase.

Lipid droplet staining

The cell cultures were fixed in 3.7% formaldehyde solution (Merck, Darmstadt, Germany) and stained with Oil red O for visualization of lipid droplets as previously described.[25]

Reverse transcription-polymerase chain reaction

RNA was isolated from the cultures by using the RNeasy mini kit (Qiagen, Venlo, the Netherlands). As a reference, two different human liver samples (non-tumour parts of small liver resection samples, immediately frozen with TRIzol® reagent (Life Technologies, Carlsbad, USA) in liquid nitrogen) were included in the analyses as described.[26] These samples derived from two female patients, aged 40 and 41 years, with liver adenoma and no elevated liver damage. The patients were not on medication and had no history of drug/alcohol abuse. The procedure was in accordance with the ethical standards of the institutional committee on human experimentation and the Helsinki Declaration of 1975.

First strand cDNA preparation and subsequent real-time polymerase chain reaction were performed as previously described.[27] Transcript levels were calculated, subsequently normalized for 18S ribosomal RNA, and expressed as a percentage of the mean transcript levels of two human liver samples as described.[28]

Assessment biochemical parameters

For the assessment of functionality and cell damage, the cultures were incubated in 1 mL of test medium (HepaRG medium with 1.5 mM $^{15}NH_4Cl$ (Sigma), 2.27 mM *D*-galactose (Sigma), 2 mM *L*-lactate (Sigma) and 2 mM ornithine hydrochloride (Sigma)). Medium samples of 0.5 mL were taken after 45 min and 24 h of incubation, at termination of the test. These time points were chosen in order to acquire homogeneous samples and reproducible data. Subsequently, cultures were washed twice with phosphate-buffered saline and stored at -20 °C for total protein content analysis.

Biochemical assays

The test medium samples were analyzed for function and cell damage parameters. Human APOA1 concentrations were determined by an enzyme-linked immunosorbant assay using a polyclonal rabbit anti-human APOA1 (Merck) in a 1:500 dilution as primary antibody, a monoclonal

mouse anti-human APOA1 (Merck) in a 1:500 dilution as secondary antibody, and horseradish peroxidase-conjugated polyclonal goat anti-mouse IgG (Dako, Glostrup, Danmark) in a 1:2000 dilution as tertiary antibody. The color reaction was developed by using **o**-phenylenediamine (Sigma). Ammonia concentrations were determined using the Ammonia (rapid) kit (Megazyme International, Wicklow, Ireland). Urea concentrations were determined according to the blood urea nitrogen test (Sigma). Labeled ^{15}N urea was measured using gas-chromatography-mass-spectrometry.[29] Amino acid concentrations were measured by gradient reversed-phase high-performance liquid chromatography.[30] Amino acids that could be determined in this analysis included ornithine, taurine, citrulline and the 20 basic amino acids, except proline and cysteine. Aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) activities were analyzed spectrophotometrically using the P800 Roche Diagnostics analyzer. Triglycerides were analysed using a glycerol-3-phosphate oxidase-PAP enzymatic test (Roche Diagnostics, Basel, Switzerland). Total protein/well was measured by spectrometry using Coomassie blue (Bio-Rad, Hercules, U.S.). The function and damage parameters were established by calculating the changes in concentration in the t=45min and t=24h samples and were corrected for protein content/well. The determined biochemical activities were linear within this time frame.

Statistical analysis

Student's t-tests were used to determine statistical differences between two groups (-DMSO *vs* +DMSO and control *vs* ALF plasma exposed groups in urea enrichment and arginine consumption tests). One-Way Anova test with Tukey post-hoc analysis were used for statistical analysis of differences between three groups, *i.e.* the control, healthy plasma and ALF plasma exposed groups. Significance was reached if $p < 0.05$. SPSS 12.0.1 (SPSS Inc., Chicago, USA) was used for statistical analysis. Average values (± standard deviation) are reported.

RESULTS

Morphology of HepaRG cultures

HepaRG cultures (-DMSO and +DMSO) were exposed for 16h to healthy or ALF plasma. The composition of these plasmas is indicated in Table I. The ALF plasmas contained elevated levels of ammonia, total bilirubin, and the cell damage parameters AST and LDH.

The morphology of both cultures was equally affected upon exposure to either the healthy and ALF plasmas, compared to control cultures (Fig. 1). Plasma-exposed cells adapted a more rounded shape and lost cell-cell contacts upon exposure. We observed lipid droplet accumulation in plasma-exposed cultures, again independent on the type of plasma used, as confirmed by Oil red O staining (Fig. 2). This lipid droplet formation may either relate to the high triglyceride levels in plasma (Table I) relative to the culture medium (<0.1 mM) and/or a steatotic response to injury as commonly found in liver.[31]

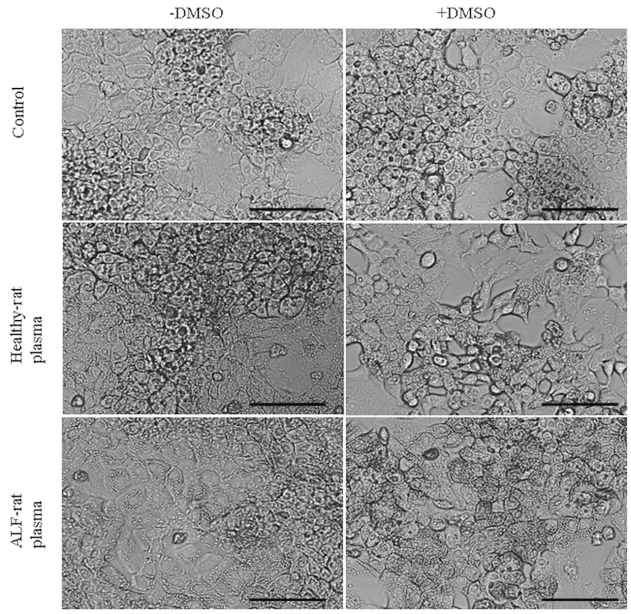

Figure 1. The effect of healthy and ALF plasma on the morphology of -DMSO and + DMSO HepaRG monolayers. Negative controls are included. Bars represent 100 μm.

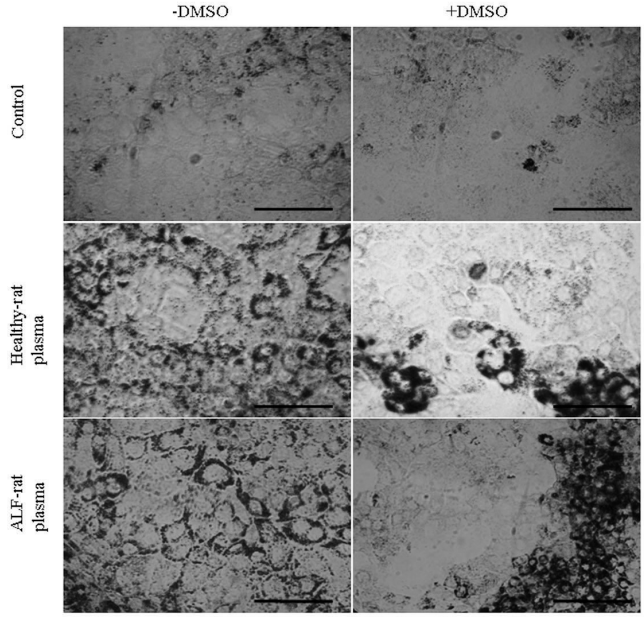

Figure 2. The effect of healthy and ALF plasma on the lipid accumulation of -DMSO and +DMSO HepaRG monolayers. Negative controls are included. Bars represent 100 μm. *For color figure, see page 219.*

Biochemical parameters of HepaRG cultures

Exposure to plasma did not change the total protein/well content substantially (Fig. 3A). As previously observed, the protein content was 2-fold lower in +DMSO cultures compared to -DMSO cultures.[6]

In contrast, cell damage, as measured by AST and LDH activities in the test medium, increased maximally 5-fold in the -DMSO cultures (Fig. 3B and C) after ALF-plasma exposure. AST leakage was also increased after exposure to healthy plasma and LDH leakage was not significantly different from control conditions. Interestingly, the AST and LDH levels remained unchanged in +DMSO cultures under all conditions, although the AST levels of +DMSO control cultures were already significantly 3 to 4-fold higher to those of -DMSO control cultures as previously observed.[6] There were no significant differences between healthy-plasma- and ALF-plasma-exposed cultures.

The ApoA1 production of the -DMSO and +DMSO cultures (Fig. 3D) was not influenced by plasma exposure and was in the same range as observed previously.[6]

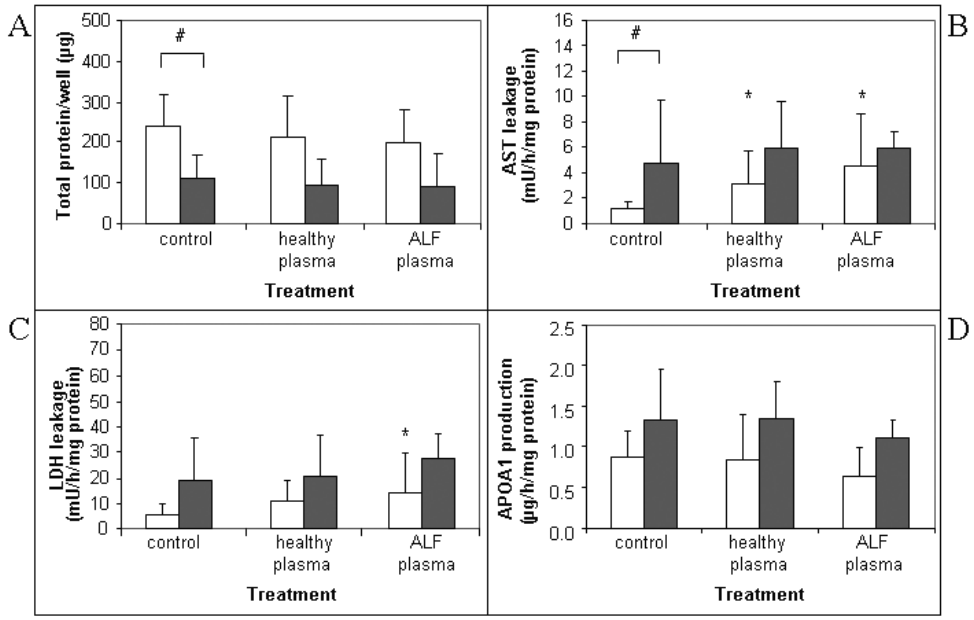

Figure 3. The effect of healthy and ALF plasma on cell mass, cell damage and APOA1 production. (A) total protein/well content, (B) AST leakage, (C) LDH leakage, and (D) APOA1 production of -DMSO (white bars) and +DMSO (gray bars) cultures. Significance: *, $p < 0.05$ *vs* control; #, $p < 0.05$ -DMSO *vs* +DMSO control cultures (n=12 from 3 independent experiments). No significant differences were present between healthy-plasma and ALF-plasma-exposed cultures after specified exposure times.

The rate of ammonia elimination (Fig. 4A) remained also constant after plasma exposure in both -DMSO and +DMSO cultures. As previously observed, ammonia elimination was 1.4-fold lower in -DMSO compared to +DMSO control cultures. Unexpectedly, the urea production (Fig. 4B) increased 2- to 3-fold in both cultures upon exposure to ALF plasma whereas no changes were observed after healthy plasma exposure. The urea production rates were similar between -DMSO and +DMSO control cultures.

To explain the discrepancy between the relatively stable ammonia elimination and, on the other hand, the increasing urea production in HepaRG cultures after exposure to ALF plasma, we determined the metabolic fate of ammonia after loading the cultures with 1.5 mM $^{15}NH_4Cl$. The mass-enriched fraction of urea (Fig. 4C) after exposure to ALF plasma was significantly reduced, namely, 6- and 4-fold in the -DMSO and +DMSO cultures, respectively. Thus, the conversion of ammonia into urea was similarly reduced in both cultures, and therefore, the ALF-plasma-associated increased urea production of both HepaRG cultures was not the result of an increase in elimination of the exogenously-added ammonia.

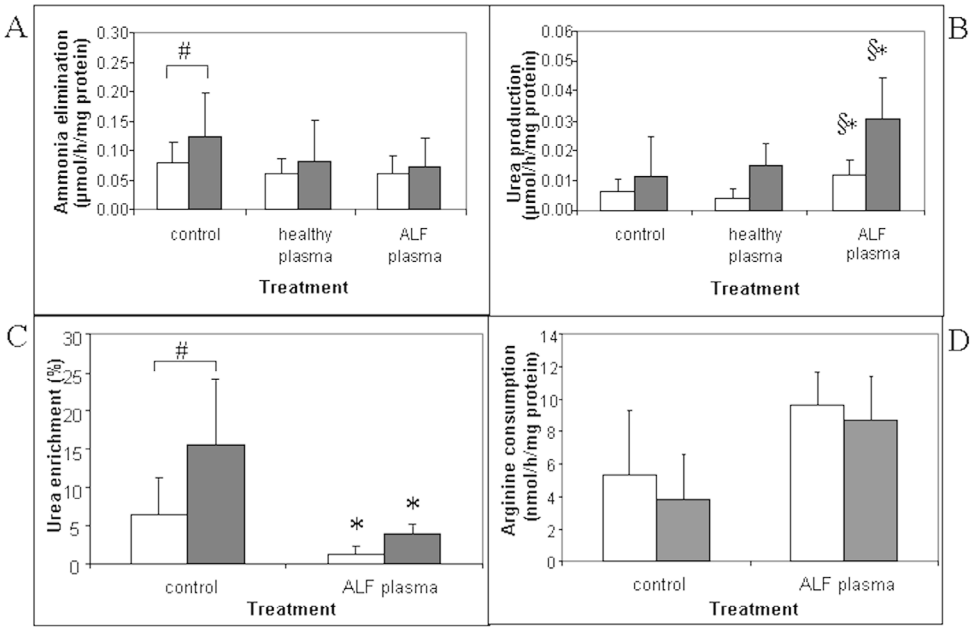

Figure 4. The effect of healthy and ALF plasmas on nitrogen metabolism. (A) ammonia elimination, (B) urea production, (C) conversion of ^{15}N ammonia into urea, and (D) arginine consumption of -DMSO (white bars) and +DMSO (gray bars) cultures For C and D only control and ALF plasma treatment groups are included. Significance: *, $p < 0.05$ *vs* control; §, $p < 0.05$ exposure to healthy plasma *vs* ALF plasma; **#**, $p < 0.05$ -DMSO *vs* +DMSO control cultures (n=12 from 3 independent experiments).

In line with these findings, the transcript levels of *CPS*, encoding the first enzyme of the urea cycle, and arginase 1 (ARG1), encoding the cytosolic urea cycle enzyme that converts arginine into ornithine and urea as part of the urea cycle, (Fig. 5A and B) remained constant or decreased in plasma-exposed cultures relative to the control cultures. Therefore, these enzymes are likely not associated with the 2- to 3-fold upregulation of urea production upon exposure to ALF plasma.

The transcript level of GS – thought to be responsible for the bulk of ammonia elimination in HepaRG cells – remained constant or decreased upon plasma exposure (Fig. 5C).[6]

Importantly, the transcript levels of *ARG2*, the cytosolic enzyme that produces urea from arginine degradation, increased 2- to 5-fold in -DMSO and +DMSO cultures after exposure to ALF plasma (Fig. 5D). These effects were due to ALF components, and not to the plasma background, as indicated by significant differences between healthy-plasma- and ALF-plasma-exposed cultures. Together, these results suggest that the increase in urea production as a consequence of exposure to ALF plasma is explained primarily by an upregulation of *ARG2*. This was further supported by a trend of increased arginine consumption in both -DMSO and +DMSO cultures after ALF plasma exposure, whereas no consistent and substantial change in metabolism of other amino acids was found (Fig. 4D).

Furthermore, we found a trend that both healthy and ALF plasmas downregulated the *CYP3A4* expression (Fig. 5E).

Finally, the differences in transcript levels of hepatic genes between -DMSO and +DMSO control cultures corresponded to a previous description of the cultures, with higher transcript levels of *CPS* and lower *CYP3A4* transcript levels for -DMSO cultures relative to +DMSO cultures.[6] No clear differences in the effect of plasma exposure on transcript levels were found between -DMSO and +DMSO cultures.

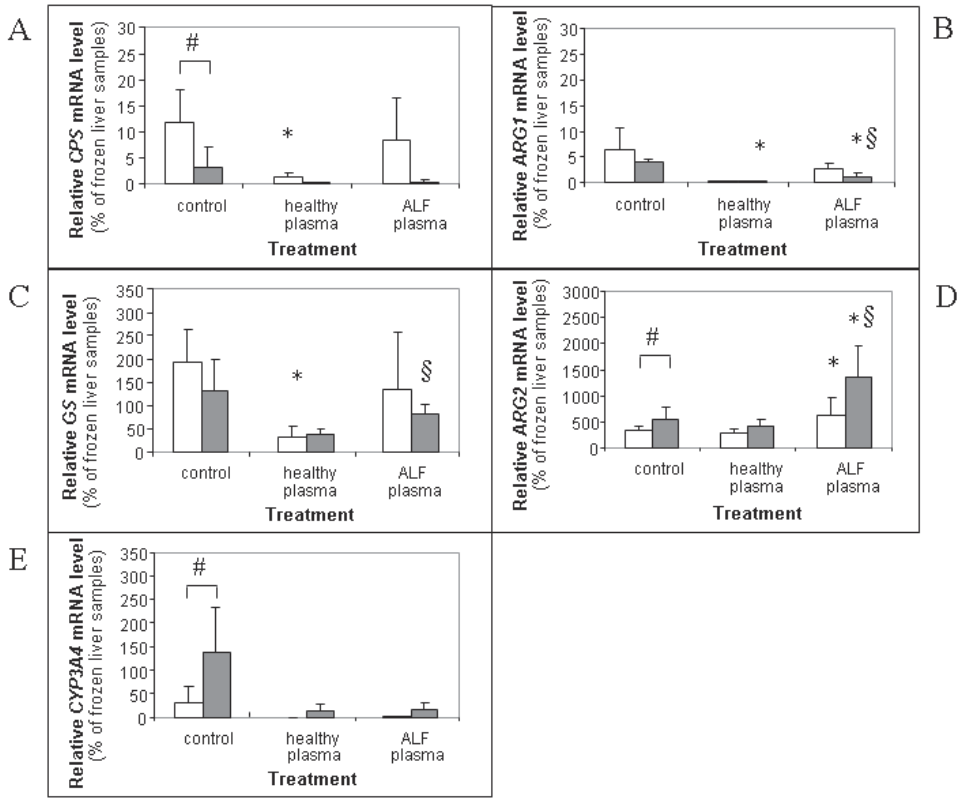

Figure 5. The effect of healthy and ALF plasmas on transcript levels of various genes. (A) *CPS*, (B) *ARG1*, (C) *GS*, (D) *ARG2* and (E) *CYP3A4* of -DMSO (white bars) and +DMSO (gray bars) cultures.. Transcript levels are indicated as the % of mean transcript levels of two human frozen control liver samples, normalized for 18S ribosomal RNA. Significance: *, $p < 0.05$ *vs* control; §, $p < 0.05$ exposure to healthy plasma *vs* ALF plasma; #, $p < 0.05$ -DMSO *vs* +DMSO control cultures (n=4 from 4 independent experiments).

DISCUSSION

This study shows the effects of healthy-rat plasma and ALF-rat plasma on different aspects of both DMSO treated (+DMSO) and non-DMSO (-DMSO) treated HepaRG cells cultured in monolayer. The cultures were both susceptible to negative effects of plasma from healthy and ALF rats. A protective effect of DMSO treatment was only found with respect to cell leakage. An as-yet unrevealed enhancing effect of ALF plasma on urea production was found, which was urea cycle-independent. Still, APOA1 production and ammonia elimination remained unaffected by exposure to plasma.

The negative effects of healthy and ALF plasma include increased cell leakage, lipid droplet formation and reduced transcript levels of various hepatic genes. The increased enzyme leakage, as a measure for cell damage, was only observed in -DMSO cultures. Since the -DMSO and +DMSO cultures differ to high extent in their detoxification characteristics, it is not unlikely that the differential cell leakage is related to detoxification capacity.[5, 32] On the other hand, the -DMSO and +DMSO cultures also differ in their metabolic activities, cell density, cell leakage and composition of cell types, which may also affect the differential response to the plasmas. Yet, upregulation of drug metabolism by DMSO treatment seems relatively ineffective in maintaining the performance of HepaRG cells after plasma exposure.[5, 6]

Surprisingly, our results imply that most of the negative effects of exposure to ALF plasma are due to the background of plasma, and not to the ALF-related composition of the plasma. The negative effects of healthy plasma may be related to the absence of beneficial compounds normally present in the HepaRG culture medium. Washizu et al. showed that the production of urea and albumin of rat hepatocytes in human plasma could be maintained or upregulated to the level in culture medium by supplementing the plasma with compounds as glucagon, amino acids, hydrocortisone and insulin.[33] Those latter two hormones are also present in non-physiological high concentrations in the HepaRG medium, rendering these as possible candidates implicated in the decline of performance after healthy-plasma exposure. In addition, amino acid concentrations may play a role, as depletion is known to lead to cell damage via autophagy, and amino acids concentrations were higher in culture medium compared to plasma.[34]

The xenogenicity of the plasmas may have contributed to the toxicity of the plasmas, although it is very unlikely that pre-existing antibodies against human liver cells are present in rat plasma. In addition, most observations on cell damage, morphology and hepatic gene transcript levels have been described for hepatocytes exposed to homologous plasma as well.[12, 15, 17, 18]

Only the increase in urea production through *ARG2* upregulation is directly caused by exposure to ALF-related components in the plasma. The physiological role and regulation of *ARG2* in the context of the liver is largely unknown, at least partly due to the high expression of *ARG1*. However in other tissues, *ARG2* and/or urea production can be upregulated by cytokines and lipopolysaccharide (LPS).[35, 36] Furthermore, *ARG2* is associated with the control of nitric oxide synthesis (a potent vasodilator involved in the inflammatory response) and the production of polyamines and proline, which are required for cell proliferation. Therefore, *ARG2* upregulation is in those tissues associated with a protective response to injury.[37] Given the fact that ALF plasma contains high levels of cytokines and LPS, a similar effect may explain the *ARG2* upregulation in HepaRG cells after exposure to ALF plasma. However, no upregulation of urea production by cytokines has been observed in primary rat hepatocytes.[38] On the other hand, HepaRG cells, with a high *ARG2* expression and a low *ARG1* expression relative to human liver, may correspond more to the extra-hepatic tissues with respect to responses to cytokines and urea production. The *ARG2* expression may also be committed to either of the

two subpopulations of the HepaRG cultures and this localization of expression may have been altered after exposure to ALF plasma.

The ammonia eliminating capacity of the HepaRG cultures remained unaffected by exposure to plasma. Even in ALF plasma, despite the reduced urea cycle activity, ammonia elimination remained high, which can be explained by its predominant elimination through fixation into amino acids, particularly glutamine, rather than conversion into urea.[6] Indeed, the glutamine metabolism remained unaffected by plasma exposure. However, since *GS* transcript levels showed a tendency to decline after exposure to plasma, it can be expected that eventually ammonia elimination will be affected.

In conclusion, cytotoxic effects of ALF plasma have been found in both -DMSO and +DMSO HepaRG cultures. The declined performance is not only due to the ALF-related components of the plasma, but also to the background of the plasma itself. Therefore the maximal time that BAL devices with HepaRG cultures can be connected to ALF patients will be highly similar between patients. However, ammonia elimination, one of the most important functions of a BAL, is relatively resistant to plasma exposure as well as APOA1 production. This supports that HepaRG cells will be suitable for BAL application. When used in a BAL system, their resistance to plasma effects may be increased by DMSO pre-treatment or supplementation of beneficial compounds present in the culture medium. Nevertheless, careful monitoring of BAL functionality during treatment of a patient will be needed to establish the moment that a BAL needs to be refreshed.

ACKNOWLEDGMENTS

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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).[6] 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.[3]

Treatment of ALF rats

ALF rats were treated with HepaRG-AMC-BAls in an extracorporeal BAL system.[3] 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 $^{15}NH_4Cl$ (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.

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

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]

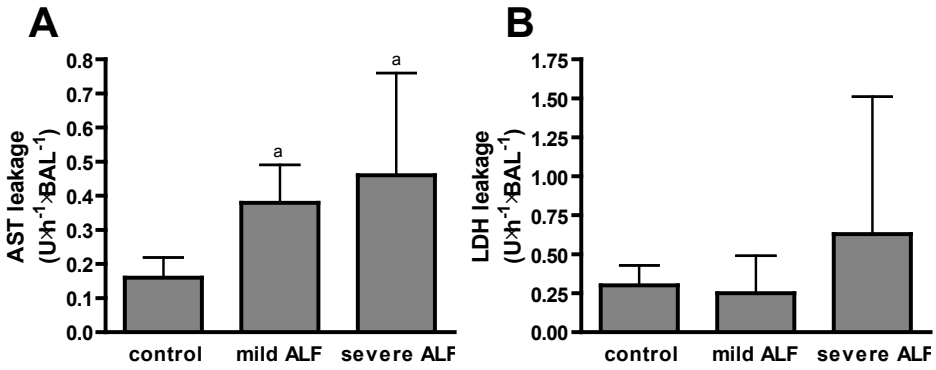

Figure 1. The effect of ALF plasma on cell damage of the HepaRG-AMC-BAL as measured by AST leakage (A) and LDH leakage (B). Abbreviations: ALF, acute liver failure; AMC-BAL, Academic Medical Center Bioartificial Liver; AST, aspartate transaminase; LDH, lactate dehydrogenase. Values are expressed as means ± standard deviations. Significance: $^{a}p < 0.05$ *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 *HNF4α (hepatic nuclear factor 4α; 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).

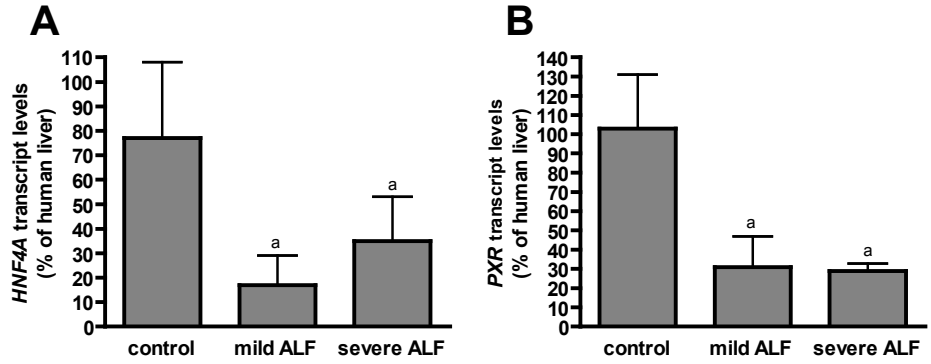

Figure 2. The effect of ALF plasma on transcript levels of hepatic transcription factors *HNF4A* (A) and *PXR* (B) of HepaRG-AMC-BALs. Abbreviations: ALF, acute liver failure; AMC-BAL, Academic Medical Center Bioartificial Liver; HNF4A, hepatic nuclear factor 4α; PXR, pregnane X receptor. Values are expressed as means ± standard deviations. Significance: $^{a}p < 0.05$ *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.

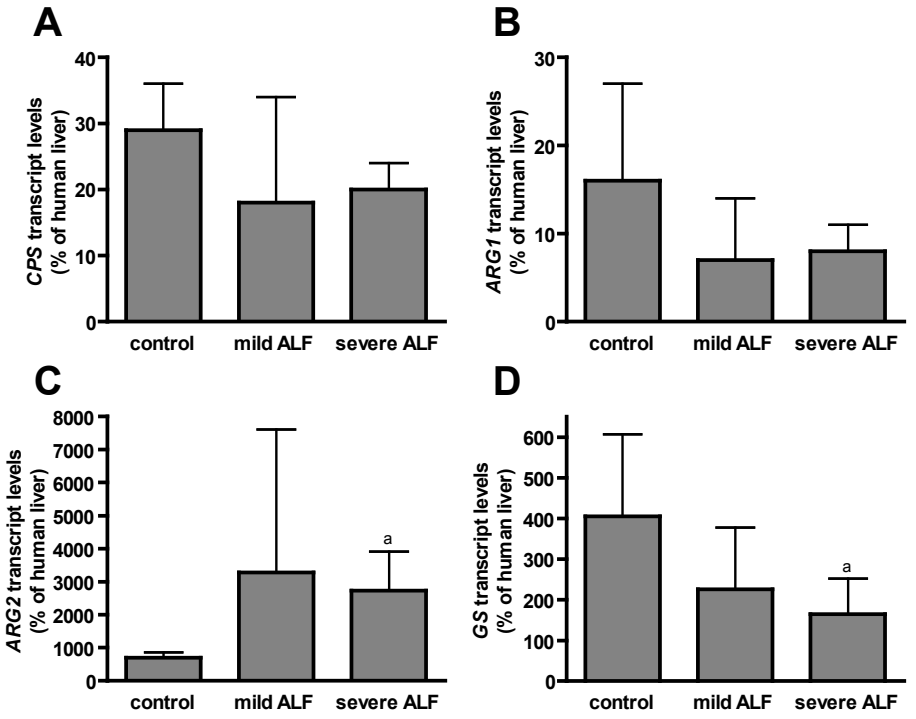

Figure 3. The effect of ALF plasma on transcript levels of hepatic genes involved in ammonia elimination and/or urea production of HepaRG-AMC-BALs: *CPS* (A), *ARG1* (B), *ARG2* (C), and *GS* (D). Abbreviations: ALF, acute liver failure; AMC-BAL, Academic Medical Center Bioartificial Liver; ARG1, arginase 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.

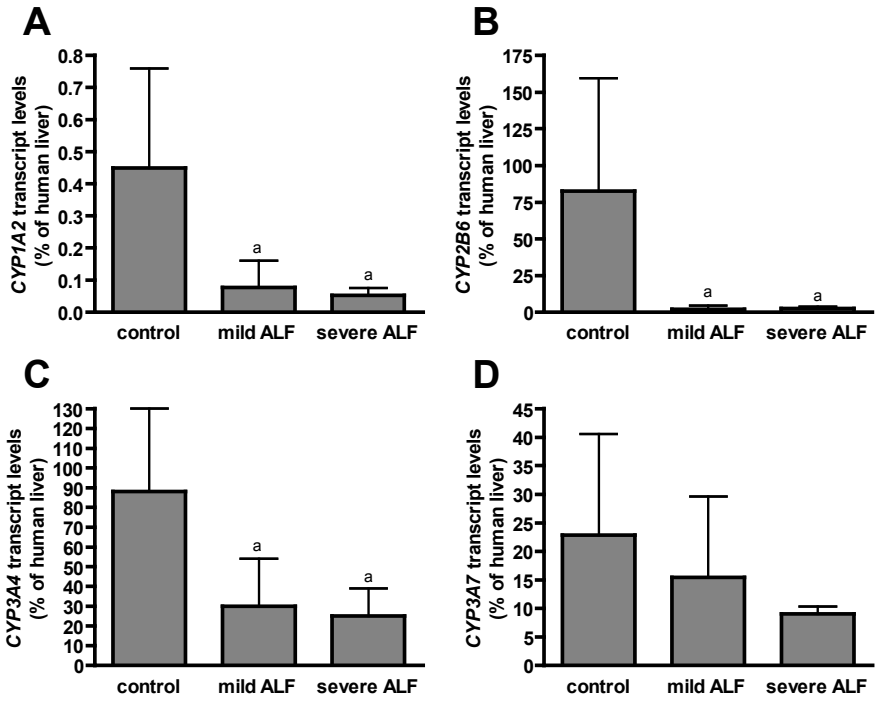

Figure 4. The effect of ALF plasma on transcript levels of hepatic enzymes involved in drug metabolism of HepaRG-AMC-BALs: *CYP1A2* (A), *CYP2B6* (B), *CYP3A4* (C), *CYP3A7* (D). Abbreviations: ALF, acute liver failure; AMC-BAL, Academic Medical Center Bioartificial Liver; CYP, cytochrome p450. Values are expressed as means ± standard deviations. Significance: $^{a}p < 0.05$ 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 ^{15}N ammonia into ^{15}N 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 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.

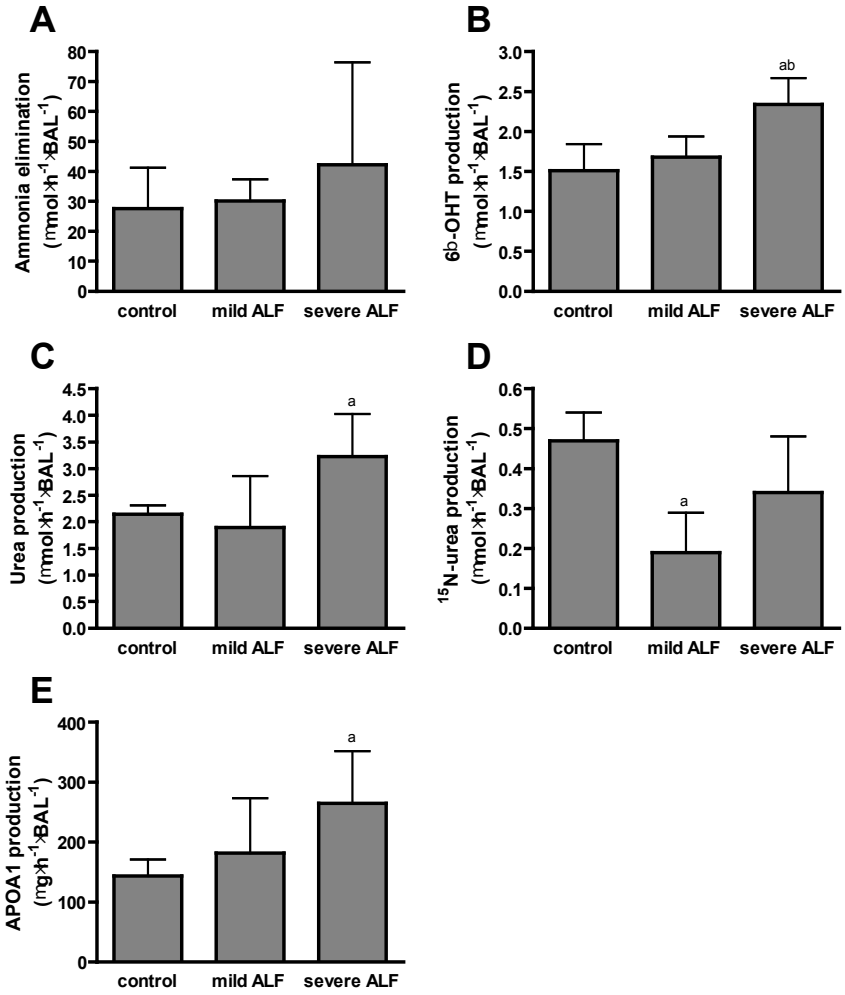

Figure 5. The effect of ALF plasma on hepatic functions of HepaRG-AMC-BALs as measured by ammonia elimination (A), 6β-OHT production (B), urea production (C), ^{15}N-urea production (D), and APOA1 production (E). Abbreviations: ALF, acute liver failure; AMC-BAL, Academic Medical Center Bioartificial Liver; 6β-OHT, 6β-hydroxytestosterone; APOA1, apolipoprotein A-1. Values are expressed as means ± standard deviations. Significance: $^{a}p < 0.05$ *versus* control group, $^{b}p < 0.05$ *versus* mild ALF group.

Table 2. Amino acid metabolism in control and ALF-plasma exposed HepaRG-AMC-BALs.

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

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). [a] $p < 0.05$ *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.[30] 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.[7] 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.[31, 32] 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.[33, 34] 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.[35, 36]

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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CHAPTER 9

Perfusion flow rate substantially contributes to the performance of the HepaRG-AMC-bioartificial liver

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ABSTRACT

Background: Bioartificial livers (BALs) are bioreactors containing liver cells that provide extracorporeal liver support to liver-failure patients. Theoretically, the plasma perfusion flow rate through a BAL is an important determinant of its functionality. Low flow rates can limit functionality due to limited substrate availability, and high flow rates can induce cell damage.

Aim: To assess the effect of flow rate through the HepaRG-AMC-BAL on its functionality.

Methods: The AMC-BAL was loaded with the liver cell line HepaRG and perfused at four different medium flow rates (0.3, 1.5, 5 and 10 mL/min).

Results: Hepatic functions ammonia elimination, urea production, lactate consumption, and 6β-hydroxylation of testosterone showed 2 to 20-fold higher rates at 5 mL/min compared to 0.3 mL/min, while cell damage remained stable. However, at 10 mL/min cell damage was 2-fold higher, and maximal hepatic functionality was not changed, except for an increase in lactate elimination. On the other hand, only a low flow rate of 0.3 mL/min allowed for an accurate measurement of the ammonia and lactate mass balance across the bioreactor, which is useful for monitoring the BAL's condition during treatment.

Conclusions: These results show that 1) the functionality of a BAL highly depends on the perfusion rate; 2) there is a universal optimal flow rate based on various function and cell damage parameters (5 mL/min for HepaRG-BAL) and 3) in the current set-up the mass balance of substrate, metabolite or cell damage markers between in-and out-flow of the bioreactor can only be determined at a suboptimal, low, perfusion rate (0.3mL/min for HepaRG-BAL).

INTRODUCTION

Bioartificial livers (BALs) have been developed to bridge patients with severe liver failure, *i.e.* acute liver failure and acute-on-chronic liver failure, to liver transplantation or liver regeneration.[1] They typically comprise a bioreactor loaded with a biocomponent that, connected to the patient's circulation, is able to replace the failing liver. The hepatic functionality of a BAL is not only dependent on the design of the bioreactor and the applied biocomponent, but also on the management of the BAL system during treatment.

The plasma flow rate through the BAL may be an important variable in this respect. Namely, the higher the flow rate, the more rapidly substrates (*e.g.* toxins) will be transported to the cells, and *de novo* synthesized proteins and other metabolic products will be transported to the patient. Thus, BAL functionality will be flow-limited at low flow rates.[2] However, at high flow rates, the limiting factor will be the intrinsic metabolic capacity of the cells inside the BAL. The intrinsic metabolic capacity comprises hepatocellular uptake, metabolization and/or synthesis, and hepatocellular export, and therefore depends on uptake- and export-receptor affinities and enzymatic reaction kinetics.

Theoretical models describe the effect of flow rate on the clearance of toxins during extracorporeal BAL support.[2, 3] Using variables such as the intrinsic metabolic capacity for a particular hepatic function (*e.g.* the clearance of a toxin), the substrate concentration at the biocomponent's cell membrane, the fraction of free *versus* plasma-protein-bound substrate, the volume of the circulating plasma/medium, a clear positive correlation was found between the flow rate and reaction kinetics of the modeled hepatic function.

However, high flow rates also generate shear stress, which potentially induces cell damage with a concurrent reduction in hepatic functionality.[4-6] Consequently, there may be an optimal plasma flow rate for every BAL that results in maximal hepatic functionality with minimal cell damage.

Despite these theoretical considerations, the impact of flow rate on hepatic functionality in a BAL has seldom been evaluated, and moreover, was only reported for a very limited number of hepatic functions. This is important, as every individual function may have its own optimal flow rate, dependant on the receptor-substrate and enzyme-substrate interactions, and their susceptibility to cell damage. In a study by Roy et al., a non-linear and plateau-reaching positive correlation was found between the de-ethylation of 7-ethoxyresofurin (cytochrome p450 1A2 activity) and flow rate, as predicted by theoretical models, however no other hepatic functions were assessed in this study.[7] Interestingly, in two other studies, such a positive correlation with flow rate could not be found for the production of albumin and urea.[5, 6] Therefore, it is unknown to what extent the flow rate differentially affects the many hepatic functions that are important for BAL treatment and whether a universal, optimal flow rate for functionality can be found.

Exposure to severe-liver-failure plasma has detrimental effects on BAL functionality, necessitating replacement of the BAL at the moment the efficacy declines.[8, 9] A monitoring system that could measure BAL functionality instantaneously would provide a valuable tool in this respect. By measuring concentrations of compounds (substrates, metabolites or cell damage markers) at the in- and out-flow of the bioreactor, a mass balance could be determined over the bioreactor, forming the basis of a monitoring system. In the past, measurements of mass balances of oxygen, ammonia and urea of hepatocyte cultures have yielded the kinetic characterization of their oxygen consumption, ammonia elimination, and urea production.[10, 11] However, it is unknown how the sensitivity of the measurement of a mass balance depends on the medium flow rate, particularly in relation with a universal, optimal flow rate for functionality.

The AMC-BAL provides a useful tool to study the effects of flow rate on various hepatic functions, as it relies on internal and equal oxygenation across the whole bioreactor and on direct cell-plasma contact (Fig. 1).[12] In contrast, most other BALs use hollow fibers to separate the cells from the plasma and rely on medium oxygenation.[13, 14] Obviously, in those systems, flow rate additionally determines the diffusion efficacy of substrates/metabolites across the membrane of the hollow fibers, as well as the oxygen supply, which hampers the accurate measurement of the direct effects of flow rate on the functionality of a BAL.[6]

When loaded with the human hepatoma cell line HepaRG, the AMC-BAL displays high hepatic functionality *in vitro*.[12, 15, 16] Furthermore, we showed that treatment with this HepaRG-AMC-BAL substantially increased the survival time in rats with acute liver failure.[16] This high functionality further emphasizes the utility of the AMC-BAL for studying the effect of flow rate on BAL performance.

Therefore, in the present study, we used the HepaRG-AMC-BAL as a model to study the effect of flow rate on various hepatic functions and cell damage parameters. In addition, we used the outcomes to define an optimal flow rate for the HepaRG-AMC-BAL. Furthermore, we studied the effect of flow rate on ammonia elimination and lactate elimination across the bioreactor. Elimination of ammonia and lactate are both important BAL functions in the treatment of patients with severe liver failure, and their concentration gradients over the bioreactor may provide a valuable tool to monitor the performance of the BAL during treatment. Importantly, both ammonia and lactate concentrations can be measured readily using electrodes at the inflow- and outflow-port of the BAL, a prerequisite to qualify as a monitoring function.

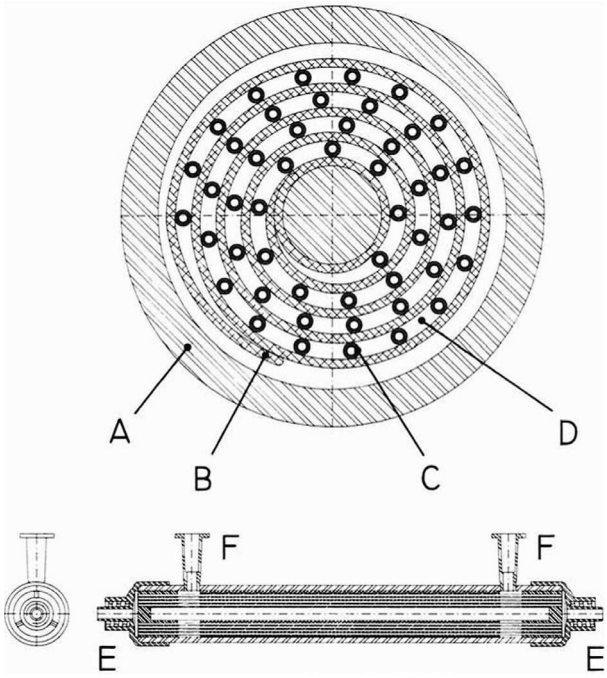

Figure 1. A schematic drawing of a transverse and longitudinal cross section of the laboratory model of the AMC-BAL. The system is composed of a polysulfone housing (A) comprising a three-dimensional non-woven polyester matrix (B) for high-density hepatocyte culture and polypropylene hollow-fibers (C) for oxygen supply and carbon dioxide removal. Plasma or culture medium is perfused through the extra-fiber bioreactor space (D) via the side ports (F) and is in direct cell contact. The bioreactor is perfused with culture gas via the endcaps (E). The hollow fibers act as spacers between the layers of the 3D matrix, creating numerous channels or extra-fiber space (D) (adapted from Flendrig *et al.*).[12]

METHODS

Cell culture

HepaRG cells were kindly provided by prof. C. Guguen-Guillouzo (INSERM, Rennes, France) and cultured and isolated as described before.[15, 16] Subsequently, HepaRG cells were cultured in laboratory-scale versions (9 mL) of third generation AMC-BALs (RanD S.r.l. Medolla, Italy) (n=5-6 per group) for 14 days (in absence of dimethyl sulfoxide) as described before (Nibourg et al., In press). In short, a suspension of 2 mL of cell pellet (approximately 750 million cells) was loaded per AMC-BAL. The cells were oxygenated with a mixture of 40% oxygen, 55% nitrogen, and 5% carbon dioxide (v/v/v) and perfused with HepaRG+CG culture medium. The HepaRG+CG culture medium was composed of Williams' E medium (Lonza, Basel, Switzerland) with 10% fetal bovine serum (Lonza), 5 μg / mL insulin (Sigma), 50 μM hydrocortisone hemisuccinate (Sigma), 2mM glutamine (Lonza), 50 U / mL penicilline and

50 μg / mL streptomycin (penicilline/streptomycine mix; Lonza), supplemented with 1mM N-carbamoyl-*L*-glutamate (Sigma Aldrich, St. Louis, U.S.) to increase urea production (Hoekstra et al 2011). The cell mass was stable during two weeks of BAL culture (Nibourg et al., In press).

Setup flow rate and functionality/cell damage tests

In the third week of culture, BALs were tested for hepatic and cell damage parameters at four different flow rates: 0.3 mL/min (n=6); 1.5 mL/min (n=6); 5 mL/min (n=6); and 10 mL/min (n=5). To this end, bioreactors were flushed with 30 mL of test medium, followed by a 2h period of recirculation with 40 mL of test medium that was composed of HepaRG culture medium supplemented with 125 μM testosterone (Sigma Aldrich, St. Louis, U.S.), 1.5 mM $^{15}NH_4Cl$ (Sigma Aldrich), 2 mM *L*-lactate (Sigma Aldrich), and 2.75 mM *D*-galactose (Sigma Aldrich) as described (Nibourg et al., In press). Samples (1 mL) were taken at 0.5, 1, 1.5, and 2h of recirculation at the inlet ports of the BALs. All samples were analyzed for concentrations of ammonia, urea, 6β-hydroxytestosterone (6β-OHT), apolipoprotein A1 (APOA1), glucose and lactate as described.[16-18] In addition, the activities of aspartate aminotransferase (AST) and lactate dehydrogenase (LDH), and the mass-enriched fraction of ^{15}N-urea were determined.[19] Function and damage parameters were determined by calculating the changes in concentration in medium per hour per BAL.

Set-up flow rate and mass balance-across-bioreactor tests

Samples (1 mL) were taken at both the inlet and outlet port of the BAL after 0.5, 1, and 2h of recirculation of test medium (see above) at flow rates of 0.3, 1.5, and 5 mL/min. Lactate and ammonia concentrations were determined as described.[18] The mass balance of ammonia and lactate across the bioreactor was calculated by subtracting the outlet-port concentration from the inlet-port concentration.

Statistical analysis

We compared differences between two experimental groups using the unpaired Student's t-test. We compared differences between three or more experimental groups using the one-way ANOVA test with Tukey's test for post-hoc comparison between two groups. 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

Effect of flow rate on hepatic functionality of the HepaRG-AMC-BAL

We assessed a number of hepatic functions in a controlled *in vitro* setting to study the effect of flow rate on hepatic functionality of the HepaRG-AMC-BAL. The capacity to eliminate ammonia, either by fixation into amino acids or, preferably, by conversion into urea is of prime importance for a BAL. Therefore, we assessed ammonia elimination, urea production, and ^{15}N-urea production after loading with 1.5 mM ^{15}N-ammonia. The ammonia elimination correlated positively with flow rate from 0.3 to 5 mL/min, with a 7-fold higher level ammonia elimination at a flow rate to 5 mL/min compared to 0.3 mL/min (Fig. 2A). The total urea production was also lowest at a flow rate of 0.3 mL/min and was 3-fold higher at the flow rates of 1.5 mL/min to 10 mL/min (Fig. 2B). In contrast to ammonia elimination, urea production did not correlate positively with flow rate beyond 1.5 mL/min. The ^{15}N-urea production was 2-fold higher at a flow rate of 1.5 mL/min relative to 0.3 mL/min, and this function remained equally high at the higher flow rates, although we observed a trend towards a decrease at a flow rate of 10 mL/min (Fig. 2C).

As a marker of hepatic detoxification (CYP3A4 activity), we determined the conversion rate of testosterone into 6β-OHT under different flow rates (Fig. 2D). At 0.3 mL/min, negligible amounts of 6β-OHT were produced; however at the flow rate of 1.5 mL/min, the 6β-OHT production was over 20-fold higher. A flow rate to 5 mL/min yielded an additionally increased the 6β-OHT production; however, the production remained stable with a flow rate of 10 mL/min.

As a marker of synthetic function, the production of APOA1 was determined. We observed no significant differences in the APOA1 production at different flow rates; however, there was a trend towards a positive correlation between function and flow rate from 0.3 mL/min to 5 mL/min, as was observed with the other hepatic functions that were assessed (Fig. 2E).

Finally, lactate elimination was assessed as a liver-specific function that prevents lactic acidosis. Lactate elimination correlated positively with flow rate, with maximally a 2.5-fold higher elimination at a flow rate of 10 mL/min compared to 0.3 mL/min (Fig. 2F).

In conclusion, there was a clear positive correlation between flow rate and functionality. However, the relative dependence of flow rate varied between the investigated functions. Except for the lactate elimination, we observed a trend towards a decrease in all other functions in bioreactors perfused at 10 mL/min relative to 5 mL/min.

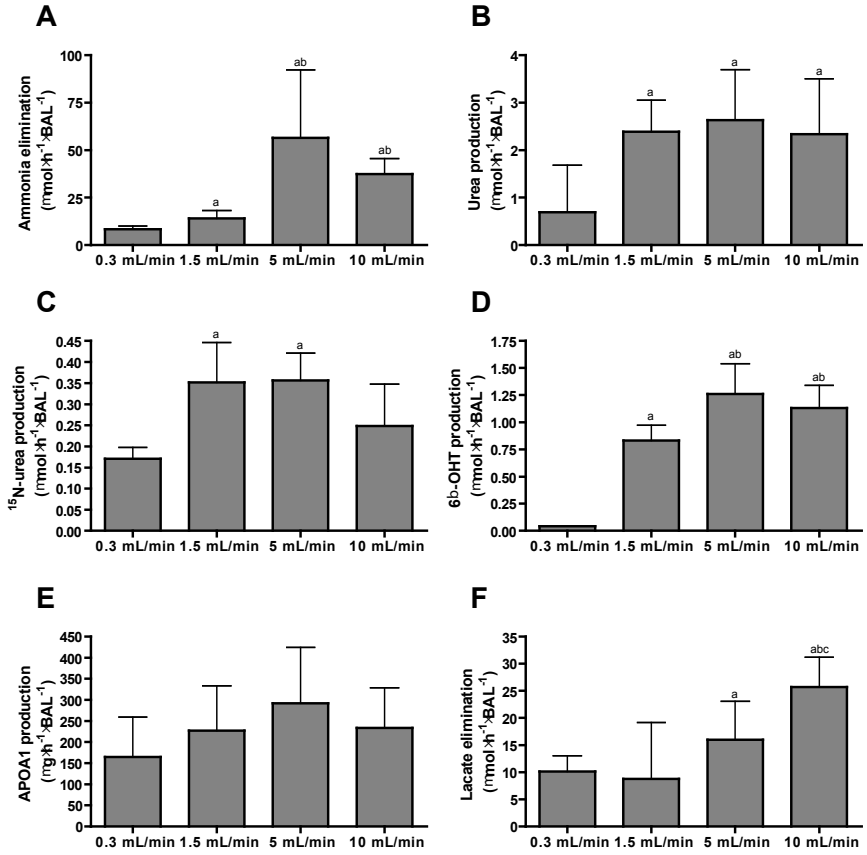

Figure 2. Hepatic functions of the HepaRG-AMC-BAL at different culture medium flow rates. Hepatic functions measured were ammonia elimination (A), urea production (B), ^{15}N-urea production (C), 6β-OHT production (D), APOA1 production (E), and lactate elimination (F). Values are expressed as means ± standard deviations. Significance: $^{a}p < 0.05$ *versus* 0.3 mL/min group, $^{b}p < 0.05$ *versus* 1.5 mL/min group, $^{c}p < 0.05$ *versus* 5 mL/min group.

Effect of flow rate on cell damage in the HepaRG-AMC-BAL

To assess cell damage at different flow rates, we measured AST and LDH leakage. In a recent study, we demonstrated that at a flow rate of 5 mL/min, AST and LDH leakage from HepaRG-AMC-BALs reaches to ~2-3% of their total cellular contents in 24 hours[16]. In this study, we found comparable AST and LDH leakage.

We observed no differences in AST or LDH leakage between BALs perfused at 0.3, 1.5, or 5 mL/min rate (Fig 3A, B). However, a flow rate of 10 mL/min doubled the AST and LDH leakage compared to the other tested flow rates, which is suggestive for shear stress induced cell damage.

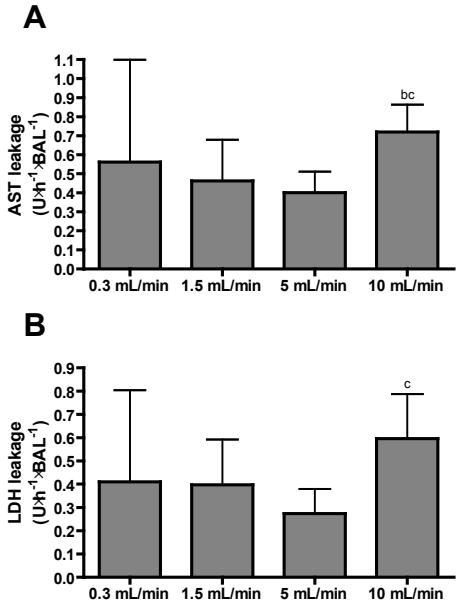

Figure 3. Cell damage measured by AST release (A) and LDH release (B) in the HepaRG-AMC-BAL at different culture medium flow rates. Values are expressed as means ± standard deviations. Significance: $^{b}p < 0.05$ *versus* 1.5 mL/min group, $^{c}p < 0.05$ *versus* 5 mL/min group.

Effect of flow rate on ammonia and lactate mass balance across the bioreactor

We measured ammonia and lactate concentration difference across the bioreactor at flow rates of 0.3, 1.5, and 5 mL/min, to evaluate their potential in monitoring BAL condition during the treatment of severe liver patients. At medium flow rates of 1.5 and 5 mL/min, no measurable amounts of ammonia and lactate were eliminated across the bioreactor, rendering these flow rates inadequate for BAL condition monitoring (Fig 4A, B). However, at a flow rate of 0.3 mL/min, ammonia and lactate mass balances were substantial at all measured time points and differed significantly from those at flow rates of both 1.5 and 5 mL/min. In addition, the mass balance of lactate and ammonia across the bioreactor remained stable in time. These results show that both ammonia and lactate mass balance are applicable as parameters to monitor BAL condition, however only at a flow rate of 0.3 mL/min in this BAL culture setup.

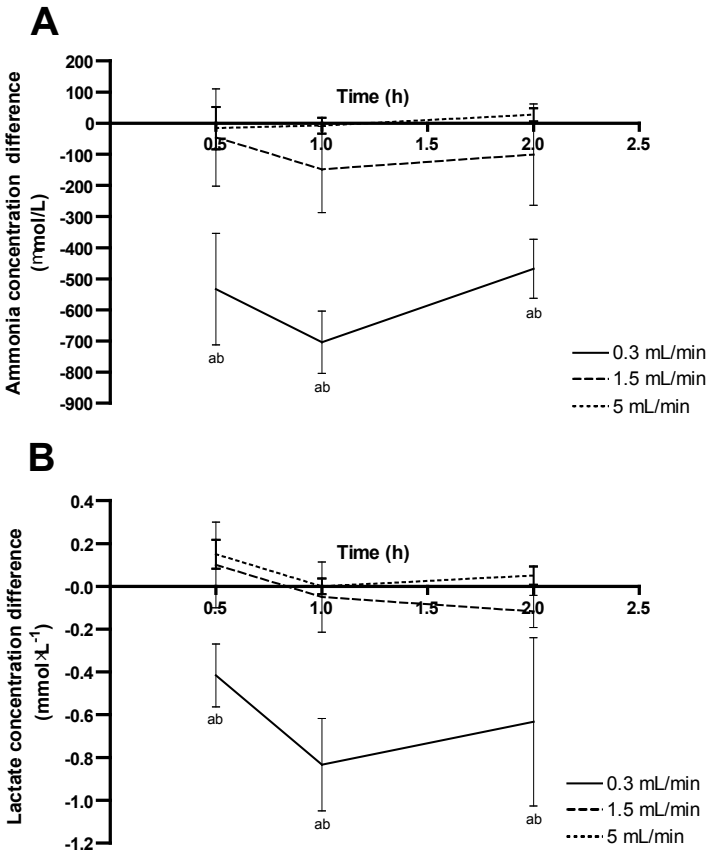

Figure 4. Concentration differences of ammonia (A) and lactate (B) across the bioreactor (outlet- minus inlet-concentrations). Values are expressed as means ± standard deviations. Significance: [a]$p < 0.05$ *versus* 1.5 mL/min group, [b]$p < 0.05$ *versus* 5 mL/min group.

DISCUSSION

Optimizing the plasma flow rate through a BAL drastically influences its hepatic functionality and thereby its efficacy. In this study, we demonstrate that the optimum medium flow rate in the laboratory scale version of the HepaRG-AMC-BAL, that has recently proved efficacious in a rat model of acute liver failure, differs per assessed hepatic function. Nonetheless, we found an overall optimal medium flow rate of 5 mL/min, based upon a maximum of hepatic functionality with a minimum amount of cell damage. In addition, we show in this study that ammonia and lactate elimination can be used as monitoring parameters of BAL condition during treatment, enabling accurate timing of BAL replacement. To our knowledge, this is the first study showing

the impact of flow rate on multiple hepatic functions in the absence of oxygen gradients and mass-transfer barriers; and also the first study that measures these hepatic functions both in time, as well as momentarily across the bioreactor.

As argued in theoretical models, our study demonstrates that hepatic functions increase with flow rate until a plateau is reached, possibly because the intrinsic metabolic capacity of the BAL becomes the limiting factor.[2, 3] In addition, we showed that the flow rate at which this maximal capacity is reached differs per hepatic function. We found a 2 to 20-fold increase in functionality at a medium flow rate of 1.5 mL/min relative to 0.3 mL/min, as measured by an increase in ammonia elimination, urea production, ^{15}N-urea production, 6β-OHT production, and lactate elimination; whereas of these functions, only ammonia elimination, 6β-OHT production, and lactate elimination rates further increased with medium flow rate from 1.5 to 5 mL/min. Furthermore, only lactate elimination continued to increase with flow rate until 10 mL/min.

When subsequently defining an overall optimal flow rate, the relative contribution of the individual hepatic functions in the treatment of severe liver failure should be taken into account. In this respect, accumulation of ammonia and other neurotoxins are of paramount importance in the pathophysiology of hepatic encephalopathy, a life-threatening complication of severe liver failure [20]. Therefore, emphasis should be put on ammonia elimination and cytochrome p450 dependent drug metabolizing activity (measured by the 6β-OHT production rate), when assessing the overall optimal flow rate. As both ammonia elimination and 6β-OHT production were maximal at 5 mL/min, and this flow rate was not associated with increased cell damage, we conclude that the overall optimal medium flow rate is 5 mL/min in our setup.

In this respect, it is noteworthy that the flow rate of the culture medium for bioreactor culturing in the 14-days prior to the tests was also 5 mL/min. Therefore, one may speculate that the HepaRG cells had adapted their receptors/enzymes relevant for specific functions, determined during the tests, to this flow rate. However, this could only be valid for APOA1 production, as the substrates for all other functions tested (ammonia, testosterone, and lactate) were only present in the test medium, and not in the culture medium.

The plasma flow rate through a BAL can be limited by the capacity of the applied plasmapheresis modules that generate the plasma perfused through the bioreactor. This problem has also been described in theoretical models for toxins of which the intrinsic clearance of the BAL is high [3]. To overcome this limitation, we and others introduced a separate high-flow circuit through the bioreactor that does not depend on the maximum plasmapheresis capacity.[21, 22]

As the cross-sectional micro-architecture of the up-scaled patient size AMC-BAL is similar to the laboratory scale AMC-BAL, the optimal medium flow rate of 5 mL/min can be extrapolated to a patient-size AMC-BAL for clinical application. To this end, we calculated the optimal flow speed along the HepaRG cells in the laboratory scale AMC-BAL. Using the specifications listed in Table I, the medium flow of 5 mL/min was divided by the internal open surface of 48.6 mm^2 of the laboratory scale AMC-BAL, rendering an optimal medium flow speed of 1.7 mm/s.

Calculating the optimal flow rate for the patient size AMC-BAL from this medium flow speed renders a theoretically optimal medium flow rate of 200-250 mL/min. Of note, the patient-size AMC-BAL used for this calculation is the third generation AMC-BAL with a thinner matrix and double the amount of oxygen capillaries compared to the second generation AMC-BAL.[21, 23]

Table 1. Specification of laboratory scale and patient size third generation AMC-BALs.

	Laboratory scale AMC-BAL	Patient size AMC-BAL
BAL opening diameter	13 mm	79.6 mm
Core diameter	3 mm	25 mm
Capillary diameter	0.38 mm	0.38 mm
Number of capillaries	195	6960
Cross-sectional matrix length	52 mm	100 mm
Length of circular matrix	300 mm	8008 mm
Thickness of matrix	0.183 mm	0.183 mm

However, a few considerations should be made regarding this optimal flow speed of 200-250 mL/min for the patient size AMC-BAL. Firstly, the medium flow through the AMC-BAL is probably not completely homogenous and depends on the BALs size. In addition, the patient size AMC-BAL is equipped with a longer matrix resulting in a more drastic depletion of substrates across the bioreactor. Furthermore, the culture medium differs from the plasma of patients with severe liver failure in composition, probably influencing functional kinetics and thereby the optimal flow speed. And lastly, plasma has a higher viscosity than the culture medium we used, as it contains a higher concentration of proteins, which increases shear stress and thereby cell damage. Therefore, the calculated optimal plasma flow rate through the patient size AMC-BAL is an estimation that still needs to be confirmed experimentally. Nonetheless, in a phase I trial conducted in 2002 with the AMC-BAL loaded with freshly isolated porcine hepatocytes, a plasma flow rate of 150 mL/min was used, which, based upon the results in this study, may have been suboptimal.

As BAL functionality will deteriorate due to the effects of the severely toxic liver failure plasma, monitoring BAL condition across the bioreactor during treatment is a valuable tool to accurately assess the timing of BAL refreshment. This study provides the proof of principle that ammonia and lactate elimination are suitable for this purpose. Ammonia and lactate concentrations can be measured quickly and easily using commercially available tests.[24, 25] Alternatively, continuous measurement of both ammonia and lactate is possible using micro-dialysis.[26] Notably, we only observed measurable concentration differences across the bioreactor at the lowest medium flow rate of 0.3 mL/min in our setup, whereas the flow rate for optimal functionality was 5 mL/min. For clinical application, it may therefore be necessary to temporarily decrease the plasma flow rate to monitor BAL functionality during treatment. The mass-balance over the bioreactor, measured under conditions of low flow rate, is therefore not reflecting the maximal

functionality of bioreactor. However the stability of the mass balance, repeatedly measured at low flow rate will be indicative for the condition of the bioreactor during treatment. Ideally, the flow for optimal functionality and mass balance measurements are equal. This may be achieved by increasing the capacity of the bioreactor or by elongating the housing of the bioreactor.

In conclusion, we demonstrate in this study that hepatic functionality of the HepaRG-AMC-BAL is highly dependent on flow rates, with optimal flow rates differing for various hepatic functions, and in our setup, an overall optimal flow rate of 5 mL/min. These results emphasize the importance of flow rate on hepatic functionality of a BAL and encourage further research to determine the optimal plasma flow rate for clinical application of the HepaRG-AMC-BAL, and for other BAL systems that are being developed as well. In addition, we provide the proof of principle for the use of ammonia and lactate elimination as parameters to monitor BAL condition during treatment.

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CHAPTER 10

Substantial phase 1 and phase 2 drug metabolism and bile acid production of HepaRG cells in a bioartificial liver in absence of dimethyl sulfoxide

Submitted

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ABSTRACT

Background: The human liver cell line HepaRG has been recognized as a promising source for *in vitro* testing of metabolism and toxicity of compounds. However, currently the hepatic differentiation of these cells relies on exposure to dimethylsulfoxide (DMSO), which, as a side-effect, has a damaging effect and represses an all-round hepatic functionality. The AMC-bioartificial liver (AMC-BAL) is a 3D bioreactor that has previously been shown to upregulate various liver functions of cultured cells.

Aim: To culture HepaRG cells in the AMC-BAL without DMSO and characterize drug metabolism.

Methods & Results: Within 14 days of culture, the HepaRG-AMC-BALs contained highly polarized viable liver-like tissue with heterogeneous distribution of cytochrome P450 (CYP) 3A4. We found a substantial metabolism of the tested substrates, ranging from 26% (UDP-glucuronosyltransferase 1A1), 47% (CYP3A4) to 240% (CYP2C9) of primary human hepatocytes. The CYP3A4 activity could be induced 2-fold by rifampicin, while CYP2C9 activity remained equally high. The HepaRG-AMC-BAL produced bile acids at 43% the rate of primary human hepatocytes and demonstrated hydroxylation, conjugation, and transport of bile salts.

Conclusions: Culturing HepaRG cells in the AMC-BAL yields substantial phase 1 and phase 2 drug metabolism, while maintaining high viability, rendering DMSO addition superfluous for the promotion of drug metabolism. Therefore, AMC-BAL culturing makes the HepaRG cells more suitable for testing metabolism and toxicity of drugs.

INTRODUCTION

The demand for safety testing of newly developed drugs rises due to stricter regulatory demands for market approval, as well as the increasing number of compounds to be tested. A large share of tests target the metabolism and toxicity of compounds in the liver, as the liver is the primary site for drug metabolism and hepatocellular and/or cholestatic liver injury is a major cause of attrition in (pre-)clinical drug development.[1]

Hepatic drug metabolism entails a myriad of chemical reactions that are also involved in the metabolism of endobiotics, such as bile acids and bilirubin. Two phases are recognized: phase 1, the basic alteration of structures predominantly catalyzed by cytochrome P450 (CYP) enzymes, and phase 2, the conjugation of a hydrophilic moiety to the drug by transferases, such as UDP-glucuronosyltransferases (UGT) and sulfotransferases. In addition, transport processes are relevant, both for uptake and excretion into either bile or back into the circulation.

The HepaRG cell line is recognized as a suitable resource for testing hepatic metabolism and toxicity of compounds.[2] The HepaRG cell line is a liver progenitor cell line that forms hepatocyte-like clusters surrounded by biliary epithelial-like cells within 28 days.[3, 4] Drug metabolism is upregulated when the culture is exposed to 2% dimethylsulfoxide (DMSO) during the last 14 days. DMSO-treated HepaRG cultures functionally express hepatic drug transporters, show phase 1 metabolic activity, with unique high CYP3A4 activity, expressed by the hepatocyte clusters [3] and high transcript levels of genes encoding phase 2 enzymes.[5, 6] Moreover, CYP1A2, 2B6, 2C9 and 3A4 activity can be upregulated by their prototypical inducers and carcinogens and acetaminophen elicit hepatotoxic responses, related to that of primary human hepatocytes (PHHs).[7-11]

However, DMSO treatment increases cell death of the HepaRG cultures; the treatment induces a more than two-fold increase in cell leakage with loss of cell mass, which may limit the sensitivity of hepatotoxicity tests.[12] In addition, DMSO represses various hepatic functions, *e.g.* the elimination of galactose, which may impair the usefulness of DMSO-treated HepaRG monolayers as a model for human liver.[12]

Culturing HepaRG in 3D in a bioreactor, the AMC-bioartificial liver (AMC-BAL) for only 14 days without DMSO generated viable cultures mimicking the human liver to high extent: the HepaRG-AMC-BALs eliminated ammonia and lactate, and produced Apolipoprotein A-1 at rates comparable to freshly isolated hepatocytes.[13] Moreover, CYP3A4 transcript level was high with 88% of the level of PHHs. The transcript levels of nuclear receptor genes *hepatocyte nuclear factor 4* and *pregnane X receptor*, both central players in the regulation of drug metabolism were between 100-150% of human liver.[14] Furthermore, a study in rats with acute liver failure showed that the HepaRG-AMC-BAL replaced the liver function leading to increased survival.[13]

The aim of this study was to further characterize the HepaRG-AMC-BAL for the metabolism of endobiotics (testosterone, bilirubin, bile acids) and a xenobiotic (tolbutamide) to explore its

suitability as a tool in compound safety studies. In addition, we studied the inducibility and localization of detoxification enzymes. Finally, we assessed bile acid production and composition, to determine the occurrence of hydroxylation, conjugation, and transport of bile acids, not only relevant for drug metabolism, but also to investigate the possibility that the HepaRG-AMC-BAL may serve as a model to study cholestasis-inducing activity of drugs.

METHODS

Biological materials and culture conditions

HepaRG cells were kindly provided by prof. C. Guguen-Guillouzo (INSERM, Rennes). At day 0, laboratory models (9 mL) AMC-BALs were loaded with ~750 million cells and cultured in 500 mL recirculating HepaRG-CG medium, *i.e.* HepaRG medium without DMSO, but supplemented with 1 mM N-carbamoyl-L-glutamate (Sigma Aldrich, St. Louis, U.S.).[13]

As a reference, PHHs were isolated at day 0 from liver tissue of patients who underwent a partial hepatectomy as described.[15] The procedure was in accordance with the ethical standards of the institutional committee on human experimentation and after obtaining informed consent. PHHs were seeded in HepaRG-CG medium into 24-well Primaria™ plates (Becton Dickinson, Franklin Lanes, U.S.) at a cell density of 2.5 x 10^5 cells/cm^2. All monolayer cultures were kept at 37ºC in a humidified atmosphere (95% air, 5% CO_2).

Immunohistochemical analysis

Transverse 8 μm sections of formaline-fixed and paraplast-embedded BALs were obtained as described.[16] Immunostaining for CYP3A4 was performed using a rabbit anti-human CYP3A4 antibody (1:100, Fitzgerald Industries International, Acton, U.S.) and alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G (1:200, Sigma). Immunostaining for multidrug resistance-associated protein 2 (MRP2) was performed using mouse anti-MRP2 antibody M2II6 (1:200) and alkaline phosphatase-labeled goat anti-mouse immunoglobulin G (1:100, Sigma).[17] The reactions were developed with 5-bromo-4-chloro-3'-indolyphosphate p-toluidine and nitro-blue tetrazolium chloride (Roche, Penzberg, Germany).

Detoxification activity tests

At day 11 bilirubin glucuronidation capacity of HepaRG-AMC-BALs was measured (UGT1A1 activity). Subsequently, bioreactors were perfused with 500 mL recirculating HepaRG-CG medium in absence or presence of 10 μM rifampicin (Sigma). At day 14, the HepaRG-AMC-BALs were tested for tolbutamide 4-hydroxylation (CYP2C9 acytivity) (test of 3 hrs), then reperfused with the 500 mL HepaRG-CG +/- rifampicine culture medium for 3 hrs and then tested for 6β-hydroxytestosterone (6β-OHT) production (CYP3A4 activity) during 2 hrs.

The detoxification activity tests were performed by connecting the BALs to 40 mL culture medium including a single test compound, *i.e.* either 100 μM tolbutamide (Sigma), 125 μM testosterone (Sigma, 200x) or 10 μM bilirubin (Sigma). The BALs were flushed with 20 mL of the medium and the remaining medium was recirculated. Samples of 0.5 mL were taken at 15-60 min intervals during testing. Finally, we lysed the content of the bioreactors for protein content determination as described.[18]

For comparison, PHHs were tested at day 1. After washing the cultures twice with phosphate-buffered saline (PBS, Fresenius Kabi GmbH, Graz) we added 1 mL of HepaRG medium with single test components, as indicated above. Medium samples were taken at regular intervals during 1-2 hours of incubation. Finally, all test cultures were washed twice with PBS and stored at -20°C for protein determination.

All experiments with bilirubin were performed in the dark and all samples were immediately frozen.

Test for bile salt production and conjugation

Medium samples taken from day 14 HepaRG-AMC-BALs at t=0, 2, 8 and 24h of perfusion were assessed for total bile salt content and composition. The bile salt production rate was determined for the period between 0-2h; after that the production rate declined. In addition, PHHs in monolayer at day 1 were tested for total bile salt production. The cultures were washed 2-fold in PBS and incubated in 500 μL fresh HepaRG medium. Samples of 100 μL were taken at 2h intervals until 6 h after initiation of the test. During that period the bile salt production increased linearly. As indicated above, BAL and PHH cultures were subjected to protein determination after termination of the experiment.

Biochemical assays

Concentrations of bilirubin, its mono- and di- glucuronides and 4-hydroxytolbutamide were measured by high performance liquid chromatography (HPLC). 6β-OHT concentrations were measured by HPLC coupled to mass spectrometry. Total bile salt concentrations were assessed by using the Total Bile Acids Assay Kit (Diazyme Laboratories, Poway, U.S). Bile salt composition was determined by HPLC electrospray tandem mass spectrometry.[19] Total protein concentrations were measured using the Coomassie Brilliant Blue G-250 staining kit (Bio-Rad, Hercules, U.S.).

Metabolic rates were established by calculating the concentration changes in time and were corrected for protein content per BAL or per well.

Statistical analysis

Student's t-tests were used to determine statistical differences. Significance was reached if $P < 0.05$. SPSS 12.0.1 (SPSS Inc., Chicago, U.S.) was used for statistical analysis. Average values (± standard deviation) are reported.

RESULTS

High level of polarization in heterogenous BAL culture

Immunohistochemical stainings of BAL cultures harvested at day 14 show that the HepaRG cells are viable and primarily located in the matrix of the bioreactor, circularly wound around a core. The cells heterogeneously expressed *CYP3A4*, as the intensity of cytoplasmic CYP3A4 staining varied clearly (Fig. 1). Furthermore, we did not observe a clear pattern of CYP3A4 positive clusters amidst CYP3A4-negative cells, as found for +DMSO monolayer.[3]

MRP2, a canalicular membrane transporter, was localized in the canalicular structures throughout the BAL culture (Fig. 1), indicating that the HepaRG cells were organized into a highly polarized and liver-like structure.[20]

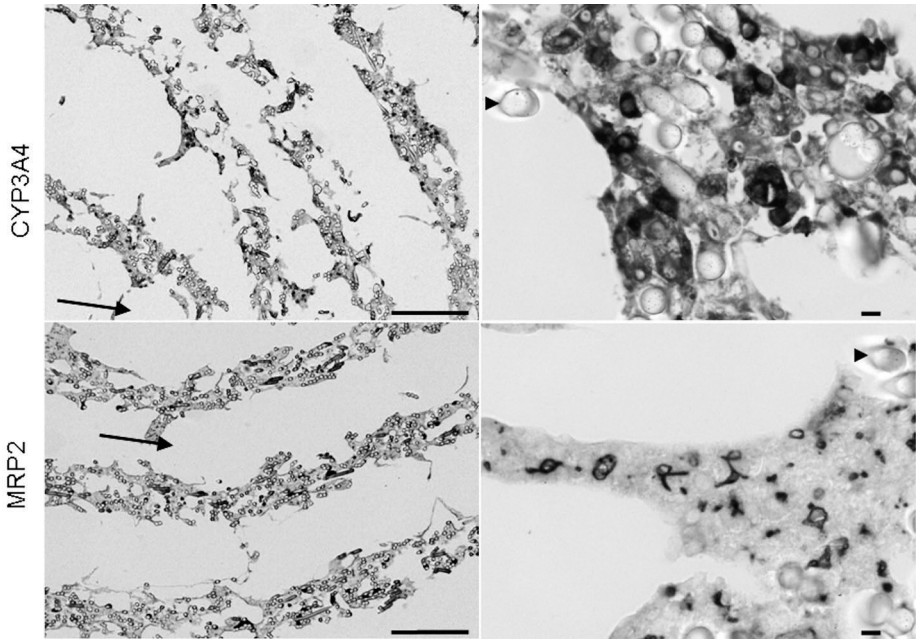

Figure 1. CYP3A4 and MRP2 stainings of cross sections of HepaRG-AMC-BALs at 14 days of culture at two different magnifications. The full transverse sections (left, bars: 500 μm) show the spirally wound matrix layers between gas capillaries (arrows show spaces left after washing off capillaries by experimental procedure) in between. Details of the matrix (right, bars: 10 μm) show the matrix fibers (arrowheads) with HepaRG cells with heterogeneous CYP3A4 distribution and canalicular MRP2 localization. *For color figure, see page 220.*

Substantial phase 1 and phase 2 drug metabolism in the HepaRG-AMC-BAL

As phase 1 drug metabolism markers, basal and rifampicine inducible CYP2C9 and CYP3A4 activities were tested in 14-day BAL cultures by measuring the hydroxylation of tolbutamide and testosterone, respectively (n=3/group) (Fig. 2A,B). The CYP2C9 activity was 0.26 ± 0.07 nmol/h/mg protein, 240% the rate of PHHs (Fig. 2A). The induction of rifampicin was not significant, although a trend towards increased CYP2C9 activity was observed. The testosterone 6β-hydroxylation, was two-fold increased by rifampicin (Fig. 2B). The basal activity was 11.2 ± 4.0 nmol/h/mg protein, 37% the rate of PHHs.

As a marker for the phase 2 enzyme UGT1A1, the rate of bilirubin glucuronidation was tested at day 11 of the BAL culture (Fig. 3D, n=6). As a reference, bilirubin glucuronidation was compared with the activity in PHHs. (n=4 for 2 isolates). Production rates of bilirubin mono-glucuronides and di-glucuronides were 44% and 15%, respectively in the BAL compared to PHHs, yielding 26% of total bilirubin glucuronidation activity.

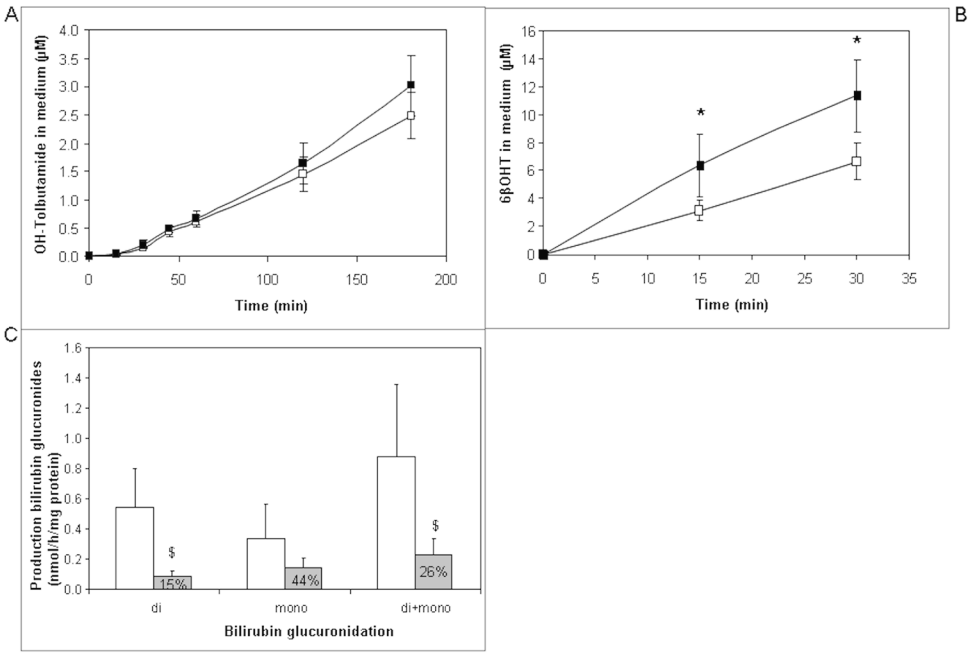

Figure 2. Phase 1 and phase 2 drug metabolic activity of the HepaRG-AMC-BALs. A) the accumulation of hydroxylated tolbutamide (CYP2C9 activity) in medium at BAL culture day 14 after a 3-days induction with 10 µM rifampicin (black squares; control without rifampicin, white squares), B), the accumulation of 6β-OHT in medium at BAL culture day 14 after a 3-days induction with 10 µM rifampicin (black squares; control without rifampicin, white squares), C) the production of mono, di and mono+di glucuronides of bilirubin of HepaRG-AMC-BALs at day 11 (grey bars) and of PHH monolayers (day 1, white bars), with the % relative to PHH monolayers given in the bars. Significance: $^{\#}$ $p < 0.05$ *versus* day 7 HepaRG-AMC-BAL, * $p < 0.05$ *versus* no rifampicin group, $^{\$}$ $p < 0.05$ *versus* PHHs.

Production of and conjugation of bile salts in the HepaRG-AMC-BAL

We performed a quantitative measurement of bile salt production and a qualitative assessment of bile salt conjugation. The bile salt production of the HepaRG-AMC-BALs (n=4) was 43% of PHH cultures (n=3-4 for 3 isolates) (Fig. 3A).

The accumulation and conjugation of specific bile salts was qualitatively determined by comparison of the HPLC mass spectra of bile salts in medium samples (Fig. 3B). The analysis was, however, hampered by the accumulation of unidentified bile acids that probably arose from the metabolism of bovine bile salts from the HepaRG medium which contains 10% (v/v) fetal bovine serum. As an example, in the chromatogram of trihydroxy bile salts we observed a stable peak for cholic acid (CA), representing a concentration of 0.05 μM and an unidentified (NI), probably non-human, bile salt peak accumulating during 24h until a concentration of 0.26 μM (Fig. 3C). The latter observation indicates that uptake and hydroxylation of bile salts occurred. Of the taurine-conjugated trihydroxy bile acids, tauro-CA (t-CA) increased from 0.1 to 0.3 μM and again an unidentified taurine-conjugated trihydroxy bile acid accumulated until a concentration of 0.1 μM. This indicates the occurrence of bile acid amidation. Of all conjugated bile salts, the accumulation of taurine-conjugated chenodeoxycholic acid (t-CDCA) was most prominent (Fig. 3D), finally making up 20% of the total bile acid pool at 24h of BAL culture. The production of dihydroxy bile salts and the conjugation of a glycine-moiety to the bile acids was negligible (data not shown).

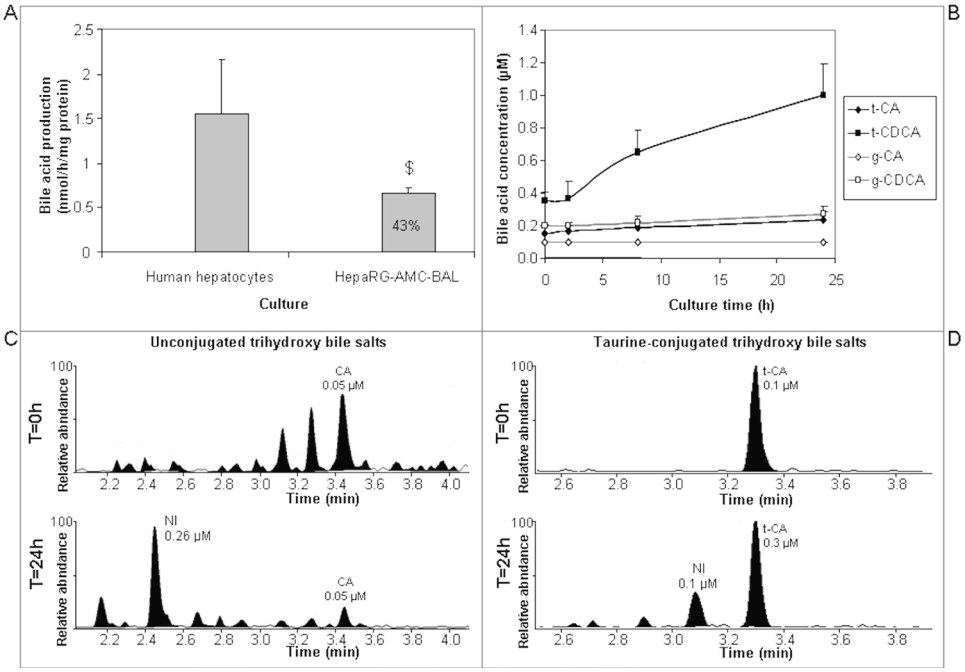

Figure 3. Production and composition of bile acids by HepaRG-AMC-BALs at day 14. A) Production of bile acids of HepaRG-AMC-BALs and of PHH monolayers (day 1), with the % relative to PHH monolayers given in the bar. Significance: $^{\$}$ $p < 0.05$ *versus* PHHs. B) the accumulation of four identified bile acids in the medium, C and D) chromatograms of unconjugated trihydroxy bile salts (C) and taurine-conjugated trihydroxy bile salts (D) of the culture medium at t=0 and t=24h. Large peaks are indicated with their concentration.

DISCUSSION

The data presented here show that the HepaRG cells in the AMC-BAL organize within 2 weeks into a highly polarized bile acid producing liver-like tissue with substantial drug metabolism, ranging from 26% (bilirubin) to 240% (tolbutamide) of PHHs. These high levels of drug metabolism have been achieved in HepaRG cells without the usage of DMSO, yielding the HepaRG-AMC-BAL a promising tool for drug safety studies targeted to the liver.

The HepaRG-AMC-BALs take up, produce, metabolize, and efflux bile acids, with a rate of bile acids accumulating in the medium of 43% of PHHs. Bile acid production and efflux is essential for hepatic signaling and for driving the bile flow.[21] Our results open the possibility to apply the HepaRG-AMC-BAL for studying the excretion of drugs or their metabolites via bile and for testing the cholestasis-inducing activity of drugs. A well-known target of drugs that induce cholestasis is the canalicular membrane transporter of primarily conjugated bile acids, named the bile salt export pump (BSEP).[22] As BSEP has a poor affinity for unconjugated

bile acids that partly constitute the bile acid pool produced by the HepaRG-AMC-BALs, it is, however, likely that bile acids will also be exported via sinusoidal transporters as Mrp3, Mrp4 and the organic solute transporter α/β.[21] However, this needs to be further confirmed, in addition to the structure of the canaliculi within in the bioreactor culture, which will also determine the destination of the bile acids.

The HepaRG-AMC-BAL has the capacity to hydroxylate bile acids, which is a CYP3A function, and to conjugate them with taurine, which is catalyzed by the enzymes bile acid CoA synthetase and bile acid-CoA:amino acid *N*-acetyltransferase.[23] The latter two enzymes are controlled by nuclear receptors farnesoid X receptor and peroxisome proliferator-activated receptor alpha.[23] These two transcription factors are also thought to play a role in the coordinate expression of *UGT* genes and hepatic transporters, as basic elements of hepatic drug metabolism. Together with the high expression of *hepatocyte nuclear factor 4* and *pregnane X receptor*, previously shown in the HepaRG-AMC-BAL, this suggests that major nuclear receptors co-ordinately regulating hepatic drug metabolism are functional in the HepaRG-AMC-BAL.

The hepatic differentiation promoting activity of the AMC-BAL is probably induced by the medium flow and the 3D organization of the culture and perhaps by the high oxygenation on site. It has been generally established that these three factors contribute to the maintenance of functionality of primary hepatocyte cultures and/or HepaRG cells, and more specifically to their drug metabolism.[24-27]

Recently, the HepaRG cells have been cultured in 3D in another bioreactor with perfusion of medium and oxygen supply on site.[28] When applying a >3 weeks BAL culture protocol including DMSO treatment, the cells showed clear drug metabolism, but no further upregulation of hepatic functions was established and even lactate production was found, whereas differentiated hepatocytes usually consume lactate.[29] Thus, culturing in 3D, with medium perfusion and oxygen supply on site is not per se sufficient for further promoting the differentiation of HepaRG cells. This may very well be attributable to the negative effects of DMSO. In a follow-up experiment DMSO was omitted in their bioreactor culture protocol.[30] The bioreactor, cultured for 5 days, was capable of biotransforming diclofenac and drug AZD6610, however the rates of substrate clearance and metabolite formation were lower compared to day 0 suspensions, indicating that their culture protocol or bioreactor still requires further improvement.

Admittedly, some characteristics of the HepaRG-AMC-BAL are also still low compared to that of PHHs, *e.g* the *CYP1A2* expression and inducibility of *CYP2C9* and *CYP3A4*.[13] To value the HepaRG-AMC-BAL as a tool for drug safety testing, we further need to explore experimental conditions, as duration of induction, concentration of the inducers, and prolonged (exceeding 14 days) culture times of HepaRG-AMC-BALs. The latter is specifically interesting, as *CYP1A2*, *2B6* and *3A4* transcript levels and CYP3A4 activity increase further after 2 weeks of BAL culturing (Nibourg et al, data not shown). In addition the metabolism of an extended set of probe substrates and the response to hepatotoxins still needs to be addressed.

On the other hand, application of the HepaRG-AMC-BAL for drug safety studies holds great promise, not only for its high and all-round hepatic functionality, but also for its long-term functionality, allowing chronic exposure experiments for >2 weeks. In addition, the medium volume can be varied over a large range, which enables either very rapid experiments (with high cell/medium ratio) or, on the other hand, chronic exposure experiments (with low cell/medium ratio) with low concentrations of drugs with high hepatic metabolism.

Concluding, culturing HepaRG cells in the AMC-BAL yields substantial phase 1 and phase 2 drug metabolism, while maintaining high viability. Therefore, and for the high flexibility in experimental set-up, HepaRG-AMC-BALs seem suitable for testing metabolism and toxicity of drugs.

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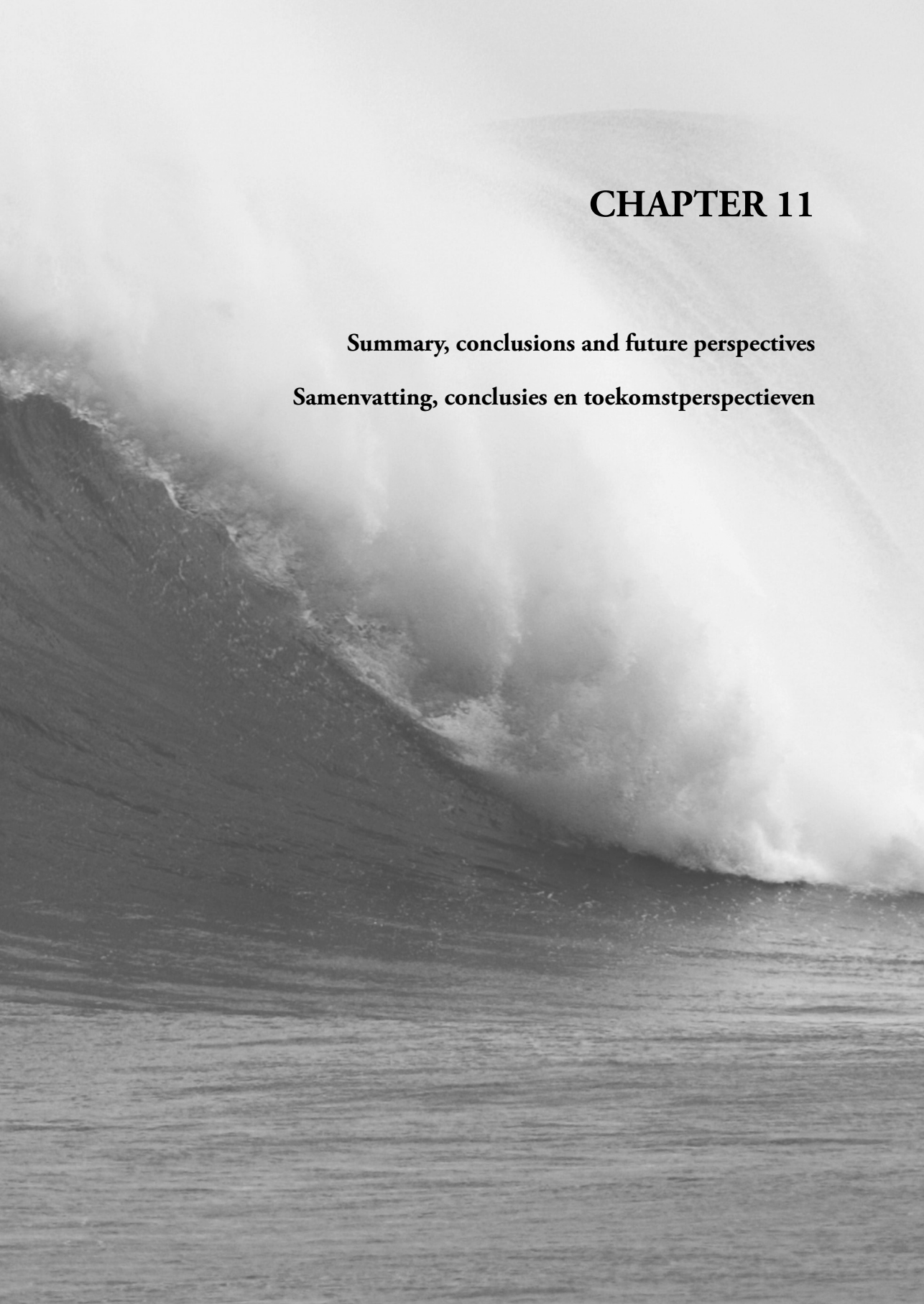

CHAPTER 11

Summary, conclusions and future perspectives

Samenvatting, conclusies en toekomstperspectieven

SUMMARY

Severe liver failure (SLF), comprising acute liver failure (ALF) and acute-on-chronic liver failure, is a syndrome with mortality rates as high as 80%.[1, 2] Clinically, the syndrome presents as a severe impairment of liver function with hepatocellular necrosis, leading to hepatic encephalopathy (HE), systemic inflammation, and multi-organ failure. Despite the progress made in supportive care, liver transplantation is often the only cure, increasing the survival rates to over 80%.[3, 4] However, liver transplantation is limited by the scarcity of donor organs. In the US, about 20% of the patients with SLF who are on the waiting list for liver transplantation die while waiting for a donor liver.[5]

Bioartificial livers (BALs) have been developed to bridge these patients to liver transplantation or liver regeneration.[6] BALs typically comprise a bioreactor that is loaded with a biocomponent with hepatic functionality that is connected to the patient's circulation. Various BAL systems, based on animal hepatocytes, are efficacious in animal models of ALF.[7, 8] However, due to xenotransplantation-related risks, there is an urgent need for BAL systems relying on human biocomponents.[9]

The main objective of the research described in this thesis was to develop such a human biocomponent for the AMC-BAL, and to test its efficacy in an animal model of acute liver failure (ALF).

Chapter 1 gives a brief overview of SLF and the AMC-BAL. It describes the etiology, pathophysiology, clinical presentation and complications of SLF. Subsequently, it addresses the treatments options for SLF, the need for effective liver replacement therapy, preferably a BAL.

In **Chapter 2** we reviewed all literature on proliferative human cell sources that have been applied in BAL systems so far and compared their performance with BALs based on primary hepatocytes. We concluded that, despite the fact that no proliferative human cell source meets the overall hepatic functionality of primary hepatocytes, the hepatoma cell line HepaRG, the hepatoma cell line HepG2-GS-CYP34 (overexpressing hepatic genes *glutamine synthetase* and *cytochrome P450 3A4*) (CYP3A4; the most dominant enzyme in hepatic drug metabolism), differentiated immature cells, *i.e.* 'small hepatocytes' and human liver stem cells, display promising *in vitro* functionality. In addition, BALs based on HepaRG cells, HepG2-GS-CYP3A4 cells, and mesenchymal stem cells have demonstrated efficacy in animal models of ALF. Lastly, various methods increasing the functionality of BALs based on proliferative human cell sources, including genetic modification, the usage of combinatory cell sources, and improvement of culture methods, hold promise, but need further assessment.

The HepG2 cell line is a widely used human hepatoma cell line that exhibits many hepatic functions. Unfortunately, its detoxification functionality (the hepatic transformation of lipophillic endo- and xenobiotics into hydrophilic excretable molecules) is low, whereas a high level of detoxification potential is a prerequisite for a BAL's biocomponent. In **Chapter 3** therefore, we evaluated the effects of stable overexpression of the *pregnane X receptor (PXR)* gene, a master regulator of detoxification, in HepG2, yielding the novel cell line cBAL119. The cBAL119 cell line showed increased transcription of several PXR target genes (49- to 219-fold) and CYP3A4 activity (4-fold) compared to the parental HepG2 cells. Importantly, this increase had not influenced its proliferation potential and several other important hepatic functions, such as its synthetic functionality. Nonetheless, the overall hepatic functionality of the cBAL119 cell line was still too low for BAL application, as *e.g.* the rate of ammonia elimination was low and inconsistent and the rate of urea production was negligible.

Therefore, we diverted focus to another human hepatoma cell line: the HepaRG cell line. This bipotent liver progenitor cell line differentiates *in vitro* into two distinct types of cells upon reaching confluence in monolayer cultures: hepatocyte-like cells and cholangiocyte-like cells. Subsequent culturing in the presence of 2% dimethyl sulfoxide (DMSO), probably by modulating the chromatin structure, further increases its detoxification function to a level that is comparable to primary human hepatocytes – so far unparalleled by any other liver cell line. Other hepatic functions that are important for BAL application, such as ammonia elimination, urea production, lactate consumption, and synthetic functionality, had not been investigated yet. In **Chapter 4** therefore, we characterized HepaRG cells cultured in monolayer, with and without the use of DMSO, and analyzed their potential for BAL application. We demonstrated in this study that HepaRG cells exert a broad array of hepatic functions, *e.g.* ammonia elimination, urea production, galactose elimination, and synthetic functionality. Unfortunately, the majority of ammonia was not converted to urea, but most likely was fixed into the amino acid glutamine, the latter being a temporary reservoir for ammonia. Interestingly, we also discovered a novel important dual effect of DMSO, while DMSO increased drug metabolism, cell damage was also increased and urea cycle activity was inhibited. Lastly, we demonstrated that ureagenesis of HepaRG cells can be increased by culturing the cells in the presence of carbamoyl glutamate, an analogue of *N*-acetylglutamate, the physiological allosteric activator of carbamoylphosphate synthetase, the rate-determining enzyme of the urea cycle under normal physiological conditions. We concluded that HepaRG cell line is a promising cell line for BAL application when cultured in the presence of carbamoyl glutamate and possibly when cultures with and without DMSO are combined.

To further study their potential for BAL application we cultured the HepaRG cells in a laboratory scale AMC-BAL, with and without DMSO as described in **Chapter 5**. HepaRG-AMC-BALs cultured without DMSO eliminated ammonia and lactate, and produced apolipoprotein A-1 (a parameter for synthetic functionality) at rates comparable to freshly isolated hepatocytes. Interestingly, DMSO treatment of HepaRG-AMC-BALs reduced the cell population and the abovementioned functions drastically. Importantly and in contrast to monolayer cultures, CYP3A4 activity was already high in HepaRG-AMC-BALs that were cultured without DMSO, and addition of DMSO did not further increase it. Therefore, solely HepaRG-AMC-BALs cultured without DMSO were tested for efficacy in rats with ALF due to total liver ischemia. HepaRG-AMC-BAL treatment increased survival time of ALF rats with 50% compared to acellular-BAL treatment. Moreover, HepaRG-AMC-BAL treatment decreased the progression of HE, kidney failure, and ammonia accumulation. These results demonstrated that the HepaRG-AMC-BAL is promising for clinical application.

The AMC-BAL provides a superior culture environment compared to monolayer, as culture in the AMC-BAL is three-dimensional, is actively oxygenated, and culture medium is constantly being perfused, as opposed to a static monolayer culture that relies on passive diffusion for oxygenation. We further studied the effects of BAL culture on the HepaRG cells as described in **Chapter 6**. HepaRG cells in the HepaRG-AMC-BAL are functionally heterogeneous, similar to monolayer cultures, and hepatic functionality of the HepaRG-AMC-BALs increased during 2-3 weeks of culture. The majority of the measured protein-normalized hepatic functions were higher in HepaRG-AMC-BALs compared to monolayer cultures, including ammonia elimination, urea production, and CYP3A4 activity. Interestingly, lactate production in monolayer cultures switched into lactate consumption in the BAL cultures, a hallmark of primary hepatocytes. In addition, protein-normalized cell damage was lower in HepaRG-AMC-BALs, and transcript levels of detoxification-associated genes and hepatic-differentiation-associated regulatory genes reached higher levels than in monolayer cultures. Lastly, metabolism of amino acids, particularly alanine and ornithine more resembled that of primary hepatocytes in HepaRG-AMC-BALs. We concluded therefore that BAL culture of HepaRG cells increases its hepatic functionality, both over time, and compared to HepaRG monolayer cultures.

During treatment of ALF patients, the biocomponent of a BAL is exposed to ALF plasma containing various toxic compounds that may, particularly after prolonged exposure, negatively affect the functionality of a BAL. In **Chapter 7**, we describe these effects on monolayer cultures of HepaRG cells, either pre-treated with DMSO or not, by exposing them to (undiluted) rat-derived ALF plasma for sixteen hours. As a control, cells were exposed to plasma of healthy rats and to the normal culture medium. Cell leakage only increased in HepaRG cultures without DMSO after exposure to plasma of both healthy and ALF rats. Exposure to rat plasma, even

of healthy origin reduced transcript levels of hepatic genes, urea cycle activity and induced lipid accumulation. Thus, the declined performance of the cells was in general not due to ALF-related components in the plasma, but to the background of the plasma itself, possibly by depletion of compounds normally present in the culture medium. Importantly, the ammonia-eliminating capacity and synthesis of apolipoprotein-A1 proved relatively resistant, underlining the suitability of HepaRG cells for BAL application.

Next, we studied the effects of rat-derived ALF plasma on the HepaRG-AMC-BAL (cultured without DMSO) as described in **Chapter 8**. We studied the effects of exposure to plasma of rats developing either mild or severe ALF. 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). Cell damage only marginally increased in both mild and severe ALF groups compared to the control group. Transcript levels of almost all hepatic genes that we studied decreased in both mild and severe ALF groups, particularly regulatory genes and detoxification-associated genes. Interestingly however, hepatic functions mostly remained unchanged in the mild ALF group, or increased in the severe ALF group. All together, the HepaRG-AMC-BAL is relatively resistant to the toxic effects of ALF plasma, with even an (probably compensatory) increase in hepatic functionality. Nonetheless, as we observed a substantial repressive effect of ALF plasma on gene transcription, longer exposure times will ultimately result in a decrease in hepatic functionality.

In **Chapter 9**, we describe the effect of medium perfusion flow rate on the functionality of the HepaRG-AMC-BAL and on the mass balance of substrates across the bioreactor. To this end we perfused laboratory scale HepaRG-AMC-BALs with culture medium at four different perfusion flow rates (0.3, 1.5, 5, and 10 mL/min) and studied the effects on hepatic functionality (ammonia elimination, urea production, lactate consumption, and CYP3A4 activity) and cell damage parameters. Overall, functionality peaked at a flow rate of 5 mL/min, being 2- to 20-fold higher compared to 0.3 mL/min. The functional differences between the 5 and 10 mL/min groups were small, but at the latter rate, cell damage increased, probably due to increased shear stress on the cells. Therefore, 5 mL/min was the optimal flow rate for the laboratory-scale BAL. When these results are extrapolated to the HepaRG-AMC-BAL for the clinical setting, the optimal flow rate should be approximately 200-250 mL/min.

In addition, we calculated mass balances of ammonia and lactate over the HepaRG-AMC-BAL with the purpose of evaluating their potential in monitoring BAL condition during the treatment of ALF patients. We demonstrated that mass balances between the inflow and outflow medium of the BAL can only be determined at suboptimal, low perfusion rate for the current set-up of the laboratory model of the HepaRG-AMC-BAL.

The HepaRG cell line has been recognized as a promising source for *in vitro* testing of the metabolism and toxicity of drugs and other compounds. However, the hepatic differentiation of these cells has so far relied on exposure to DMSO, which (as discussed above) has a damaging effect and represses all-round hepatic functionality. In **Chapter 10** therefore, drug metabolism of the HepaRG-AMC-BAL cultured without DMSO was evaluated more extensively. The HepaRG-AMC-BALs contained highly polarized viable liver-like tissue with heterogeneous distribution of CYP3A4. We found a substantial metabolism of the tested substrates, ranging from 26% (UDP-glucuronosyltransferase 1A1), 47% (CYP3A4) to 240% (CYP2C9) of primary human hepatocytes. In addition, the HepaRG-AMC-BAL produced bile acids at 43% the rate of primary human hepatocytes and demonstrated hydroxylation, conjugation, and transport of bile salts as well. These results render DMSO addition superfluous for the promotion of drug metabolism. Therefore, AMC-BAL culturing makes the HepaRG cells more suitable for testing metabolism and toxicity of drugs.

CONCLUSIONS AND FUTURE PERSPECTIVES

The major objective of this study was to develop a human biocomponent that, when cultured in the AMC-BAL, is able to effectively replace the failing liver of ALF patients. Therefore, this biocomponent needs to display a high and broad level of hepatic functionality, should be expandable without losing its functionality, and should be safe.

The studies in this thesis show that the HepaRG cell is a very promising biocomponent with respect to functionality. Namely, when cultured in AMC-BAL using a modified cultured protocol with carbamoyl glutamate and without the use of DMSO, it has demonstrated a high and broad level of *in vitro* hepatic functionality, and more importantly, treatment of ALF rats with this HepaRG-AMC-BAL substantially increased their survival time. Further analysis of the HepaRG-AMC-BAL demonstrated relative resistance to the toxic effects of ALF plasma. In addition, we assessed an optimal medium/plasma flow rate. Therefore, this preclinical work paves the path for clinical studies with the HepaRG-AMC-BAL.

Nonetheless, some important issues need to be addressed before the HepaRG-AMC-BAL can be tested in a clinical trial. First, the results of the rat study should preferably be confirmed by a second study in larger animals using a clinical scale bioreactor. Taking into account that 30-40% of functional liver mass is needed to maintain homeostasis and ALF and ACLF patients generally have some remnant liver functionality, the HepaRG-AMC-BAL should probably contain at least the equivalent of 15-20% of liver mass to be efficacious in ALF and ACLF patients.

In addition, functional stability and safety of the HepaRG-AMC-BAL should be addressed. Functional stability could be studied by prolonged culture of HepaRG cells in the AMC-BAL

with sequential functionality tests. Safety of the HepaRG-AMC-BAL encompasses toxicological and sterility studies of the HepaRG cells, the bioreactor with and without the HepaRG cells as well as the extracorporeal system as a whole. In addition, as HepaRG cells derive from a hepatocellular carcinoma and are therefore potentially tumorigenic, tumorigenicity studies need to be performed. Even if these indicate a lack of tumorigenicity (as shown in the past), fully controlled filters need to be included in the extracorporeal system to prevent potentially detached cells to enter the body.[10]

Ideally, BAL therapy should not only bridge ALF and ACLF patients to liver transplantation, but supply sufficient liver support to allow the liver to regenerate, rendering a liver transplantation superfluous. The applied rat model of ALF by inducing complete liver ischemia does not leave any remnant viable liver mass and therefore does not allow liver regeneration. Therefore, a new animal study on the effect of HepaRG-AMC-BAL treatment on liver regeneration is needed. In this respect, animal models based on a subtotal liver ischemia or a subtotal hepatectomy, optionally combined with a toxic ALF model, such as an acetaminophen overdose or a galactosamine / lipopolysaccharide injection, could be useful.

As mentioned in the general introduction of this thesis, the deleterious role of glutamine in the pathogenesis of hyperammonemia-associated HE has been identified only recently.[11] In short, ammonia is detoxified in the brain by fixation into glutamine, which is transported into the astrocyte's mitochondrion. Subsequent intra-mitochondrial hydrolysis by glutaminase locally increases ammonia levels that in turn induce oxidative stress, resulting in mitochondrial swelling and dysfunction, leading to alterations in energy metabolism, signalling mechanisms, astrocytic glutamate uptake, and cell swelling. Notably, the main route of ammonia elimination by the HepaRG-AMC-BAL is the less preferred conversion into glutamine and not the permanent conversion into urea. Therefore, the use of glutamine scavengers, such as sodium phenylacetate or sodium benzoate that form conjugates with glutamine that subsequently can be excreted by the kidneys, may reduce glutamine concentrations and thereby add in the treatment of ALF patients with the HepaRG-AMC-BAL.[12] Alternatively, improving urea cycle activity in the HepaRG-AMC-BAL, *e.g.* by overexpressing genes that encode the rate-limiting enzymes of the urea cycle, may divert the main route of ammonia elimination and thereby decrease glutamine production.

In the search for a suitable biocomponent for the AMC-BAL, we have thus far focussed on hepatocytic functional replacement as a primary goal, as hepatocytes constitute approximately 80% of the functional liver volume. Nonetheless, non-parenchymal cell types play an important role in liver function as well. The role of Kupffer-cells in ALF and ACLF, the resident macrophages of the liver that scavenge many blood-borne pathogens such as endotoxins, is far from clear. Namely, inhibition of Kupffer-cells can both aggravate or attenuate liver injury, possibly related to different functional subsets that these plastic cells can differentiate into (M1 and M2).[13] Other hepatic non-parenchymal cells such as endothelial cells and stellate cells have

demonstrated to stabilize and increase hepatocytic functionality in co-cultures, and may offer interesting possibilities for co-culture in a BAL.[14] Also, mesenchymal stem cells, multi-potent stromal cells that (among others) exert cytokine-mediated immunosuppressing effects, are an interesting candidate for co-culture in this respect.[15]
To be continued.

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SAMENVATTING

Ernstig leverfalen (ELF) wordt veroorzaakt door het verlies van meer dan circa 60% van de functionele levermassa en is een ernstig ziektebeeld met een hoog sterftecijfer van ongeveer 80%. Het ziektebeeld omvat twee syndromen: acuut leverfalen (ALF) en acuut-op-chronisch leverfalen (ACLF). Het verschil tussen beide is dat in het geval van ACLF al sprake was van een leverziekte (bijvoorbeeld levercirrose) voor het optreden van leverfalen, en in het geval van ALF niet.

De oorzaken van ALF zijn divers, maar meest voorkomend zijn virale hepatitis, paracetamol intoxicatie en overgevoeligheidsreacties op geneesmiddelen. In het geval van ACLF is het vaak een relatief milde gebeurtenis zoals een infectie die de al bestaande leverziekte doet ontsporen. ELF leidt tot verschillende levensbedreigende complicaties zoals stollingstoornissen (die weer kunnen leiden tot ernstige bloedingen), oncontroleerbare sepsis (bloedvergiftiging), multi-orgaanfalen en hepatische encephalopathie (HE). HE is een neuropsychiatrische aandoening gekenmerkt door verwardheid, desoriëntatie en bewustzijnstoornissen, die uiteindelijk leidt tot coma.[1, 2] De pathofysiologie (moleculaire mechanisme) van ELF is zeer complex en nog niet geheel opgehelderd, maar wel staat vast dat ophoping van ammoniak, dat door de lever normaliter omgezet wordt in ureum dat vervolgens in de urine uitgescheiden wordt, en van andere toxische stoffen een belangrijke rol spelen in de pathofysiologie van ELF.

Voor ELF patiënten is een levertransplantatie vaak de enige adequate behandeling, maar door een tekort aan donorlevers sterft ongeveer 20% van deze patiënten voordat er een donorlever beschikbaar is.[3-5] Behandeling van deze patiënten met een kunstlever, die de leverfunctie tijdelijk kan overnemen, zou hen kunnen overbruggen naar levertransplantatie of naar herstel van de lever.[6] Dit laatste is mogelijk omdat de lever een bijzonder goed herstellend (regeneratief) vermogen heeft. Een bioartificiële lever (BAL) is zo'n kunstlever die bestaat uit een bioreactor waarin een biocomponent gekweekt wordt, normaliter levercellen (hepatocyten). Eenmaal aangesloten op de bloedsomloop (circulatie) van de patiënt kan de BAL buiten het lichaam de leverfunctie overnemen.

De AMC-BAL is een BAL die in het Academisch Medisch Centrum (AMC) is (zie figuur 1 en 2 op blz 17 en 18) en die bestaat uit een bioreactor met daarin een interne oxygenator: de semipermeabele zuurstofcapillairen (dunne buisjes die enkel gassen doorlaten), en een spiraalsgewonden polyester mat waarin de cellen kunnen hechten. Door de ruimtes tussen de mat en de zuurstofcapillairen stroomt het plasma (bloed zonder de bloedcellen) van de patiënt. Het celtype dat gebruikt kan worden als biocomponent is een kritische stap gebleken in de ontwikkeling van de AMC-BAL.[7] Menselijke (humane) hepatocyten zouden daarom de ideale biocomponent zijn. Echter, deze zijn niet voorhanden in de grote hoeveelheden die nodig zijn om de AMC-BAL te vullen. Dierlijke hepatocyten kunnen niet gebruikt worden vanwege de risico's op afweerreacties en op het overbrengen van ziekten van dier op mens (zoönosen).

De belangrijkste doelstelling van dit proefschrift was daarom het ontwikkelen van een humane biocomponent voor de AMC-BAL die beschikbaar is in grote hoeveelheden en voldoende leverspecifieke (hepatische) functionaliteit heeft om de leverfunctie van de falende lever van ALF en ACLF patiënten tijdelijk over te kunnen nemen.

Hoofdstuk 1 geeft een kort overzicht van ELF en de AMC-BAL. Het beschrijft de oorzaken, pathofysiologie, klinische presentatie en de complicaties van ELF in meer detail dan hierboven. Vervolgens beschrijft het de behandelingsopties voor deze patiënten en de noodzaak van leververvangende therapie, bij voorkeur door een BAL.

In **Hoofdstuk 2** geven we een overzicht van alle proliferatieve (zich vermenigvuldigende) humane celtypen die als biocomponent in een BAL getest zijn. We hebben BALs met al deze celtypen qua hepatische functionaliteit vergeleken met vergelijkbare BALs met vers geïsoleerde hepatocyten, de gouden standaard. We concludeerden dat er geen enkele biocomponent is die wat betreft hepatische functionaliteit vergelijkbaar is met de vers geïsoleerde hepatocyt. Echter, de hepatoma cellijnen HepaRG en HepG2-GS-CYP3A4 (deze laatste brengt de hepatische genen *glutamine synthetase* and *cytochrome P450 3A4* tot overexpressie, waarbij CYP3A4 een van de belangrijkste enzymen in de hepatische detoxificatie (zie verder) is) en de gedifferentieerde voorloper cellen 'small hepatocytes' en humane leverstamcellen, zijn het meest veelbelovend wat betreft hun hepatische *in vitro* functionaliteit. Daarnaast hebben BALs gebaseerd op HepaRG cellen, HepG2-GS-CYP3A4 cellen en mesenchymale stamcellen laten zien effectief te zijn in diermodellen van ALF. Tenslotte laten we zien dat er verscheidene methoden zijn om de hepatische functionaliteit te verbeteren, zoals genetische modificatie, het gebruik van combinaties van cel typen, en het verbeteren van kweekmethoden.

De HepG2 cellijn is een veelgebruikte humane hepatoma cellijn die veel hepatische functionaliteit heeft, echter, de detoxificatie is slecht. De hepatische detoxificatie behelst het zodanig transformeren van lichaamseigen en lichaamsvreemde toxische moleculen dat ze uitgescheiden kunnen worden via de gal of met de urine. Deze functie is belangrijk voor een BAL omdat er anders toxische ophoping van deze stoffen ontstaat. In **Hoofdstuk 3** beschrijven we de effecten van overexpressie van het gen *'pregnane X receptor' (PXR)*, een belangrijke regulator van de hepatische detoxificatie, in de HepG2 cellijn. Deze nieuwe celllijn hebben we cBAL119 genoemd. De cBAL119 cellen lieten toegenomen transcriptie (49x tot 219x) van verschillende PXR-gereguleerde genen en CYP3A4 activiteit (4x) zien ten opzichte van de moedercellijn HepG2. De modificatie bleek geen negatieve invloed te hebben op de andere hepatische functies. Niettemin was de totale hepatische functionaliteit van de cBAL119 cellen nog te laag, aangezien onder andere de ammoniak-eliminerende capaciteit wisselend en onvoldoende was, en de productie van ureum verwaarloosbaar.

We hebben daarom het focus verlegd naar een andere humane hepatoma cellijn: de HepaRG cellijn. Deze bipotente lever voorloper cellijn differentieert *in vitro* in twee verschillende celtypen: hepatocyt-achtige cellen en galweg-achtige cellen. Het vervolgens kweken van deze cellen in aanwezigheid van 2% dimethyl sulfoxide (DMSO) verhoogt de detoxificatiefunctie verder (waarschijnlijk door het moduleren van de chromatinestructuur van het DNA) tot een niveau vergelijkbaar met vers geïsoleerde hepatocyten. Andere hepatische functies die belangrijk zijn voor BAL-applicatie zoals eliminatie van ammoniak, productie van ureum, lactaat consumptie, en synthetische functionaliteit, waren echter nog nooit onderzocht. Daarom hebben we in **Hoofdstuk 4** al deze functies onderzocht in monolayer kweken (traditionele kweek van één celllaag dik) van HepaRG cellen, met en zonder toevoeging van 2% DMSO. We laten zien dat de HepaRG cellen een breed spectrum van hepatische functies tentoonspreidt, zoals eliminatie van ammoniak, productie van ureum, galactose eliminatie, en synthetische functionaliteit. Helaas werd het merendeel van het geëlimineerde ammoniak niet omgezet in ureum, maar in het aminozuur glutamine, dat slechts een tijdelijk reservoir is voor ammoniak. Interessant genoeg ontdekten we ook dat DMSO weliswaar de hepatische detoxificatie verhoogde, maar tevens de lekkage (schade) aan de cellen, en daarnaast onderdrukte het de productie van ureum. Als laatste laten we in dit hoofdstuk zien dat de productie van ureum kon worden verhoogd door het toevoegen van de stof carbamoylglutamaat, een analoog van *N*-acetylglutamaat, de fysiologische allosterische activator van carbamoylphosphate synthetase, het snelheidsbeperkende enzym van de ureum cyclus onder normale fysiologische omstandigheden. Daarom concludeerden we dat de HepaRG cellijn een veelbelovende cellijn is voor BAL applicatie in aanwezigheid van carbamoylglutamaat, en mogelijk wanneer kweken met en zonder DMSO gecombineerd gebruikt worden als biocomponent.

Vervolgens hebben we de HepaRG cellijn gekweekt in laboratorium-versies van de AMC-BAL, wederom met en zonder DMSO, en verder gekarakteriseerd in **Hoofdstuk 5**. Deze HepaRG-AMC-BALs gekweekt zonder DMSO elimineerden ammoniak en lactaat, en produceerden apolipoproteïne A-1 (een parameter voor synthetische functionaliteit) met een snelheid die vergelijkbaar was met die van vers geïsoleerde hepatocyten Het toevoegen van DMSO aan het kweekmedium reduceerde de celpopulatie en bovengenoemde functies sterk. De CYP3A4 activiteit was, in tegenstelling tot de monolayer kweken, ook al hoog in de HepaRG-AMC-BALs die zonder DSMO gekweekt waren en DMSO verhoogde deze niet verder. Daarom hebben we de HepaRG-AMC-BALs zonder DMSO getest in ratten met ALF veroorzaakt door totale leverischemie. De behandeling van ratten met de HepaRG-AMC-BAL verlengde hun overlevingstijd met 50% ten opzichte van ratten die werden behandeld met een AMC-BAL zonder cellen (controlegroep). Daarnaast vertraagde HepaRG-AMC-BAL behandeling de progressie van HE, nierfalen, en ammoniakstapeling. Deze resultaten lieten zien dat de HepaRG-AMC-BAL een veelbelovende BAL is voor klinische toepassing.

De kweekomgeving in de AMC-BAL is superieur ten opzichte van monolayer kweken, omdat de AMC-BAL, in tegenstelling tot monolayer kweken, een driedimensionale kweekomgeving biedt, de cellen actief oxygeneert, en het kweekmedium constant perfundeert. In **Hoofdstuk 6** beschrijven we de effecten van BAL-kweek op de HepaRG cellen meer in detail. De HepaRG cellen in de AMC-BAL waren functioneel heterogeen, net zoals in de monolayer kweken. De hepatische functionaliteit van de HepaRG cellen nam gedurende de eerste twee tot drie weken van de AMC-BAL kweek toe. Daarnaast waren de meeste hepatische functies (o.a. de eliminatie van ammoniak, productie van ureum en CYP3A4 activiteit) hoger in de HepaRG-AMC-BAL ten opzichte van HepaRG monolayer kweken. Bovendien veranderde het lactaat metabolisme: van productie in de monolayer kweken naar consumptie in de BAL, en dit laatste is een belangrijk kenmerk van vers geïsoleerde hepatocyten. Ook de hoeveelheid cellekkage was lager in de BALs ten opzichte van monolayer. Transcript niveaus van genen die geassocieerd zijn met detoxificatie en celdifferentiatie waren ook hoger in de HepaRG-AMC-BALs en het aminozuurmetabolisme van de HepaRG-AMC-BALs was meer vergelijkbaar met dat van hepatocyten. Daarom concludeerden we in dit hoofdstuk dat het kweken in de AMC-BAL de hepatische functionaliteit van de HepaRG cellen verhoogt, zowel in de tijd, als ten opzichte van HepaRG monolayer kweken.

Tijdens de behandeling van ALF patiënten wordt de biocomponent van de BAL langdurig blootgesteld aan ALF plasma van de patiënt met daarin velerlei toxische moleculen die de functionaliteit van de BAL zouden kunnen verlagen. In **Hoofdstuk 7** beschrijven we de effecten van blootstelling aan ALF plasma op HepaRG cellen in monolayerkweken. HepaRG cellen, zowel met als zonder DMSO gekweekt, werden blootgesteld aan onverdund ALF plasma van ratten gedurende 16 uur. Als controle werden cellen gedurende dezelfde tijd blootgesteld aan onverdund plasma van gezonde ratten of aan normaal kweekmedium. Alleen de HepaRG cellen die zonder DMSO gekweekt waren lieten een toename in cellekkage zien als gevolg van blootstelling aan het plasma van ALF ratten, en dat gebeurde ook na blootstelling aan het plasma van gezonde ratten. Blootstelling aan beide plasma's verlaagde ook de ureumcyclus activiteit, induceerde vetstapeling in de HepaRG cellen, en verlaagde ook de transcript niveaus van de meeste geteste hepatische genen. De verminderde activiteit van de cellen werd dus in het algemeen niet veroorzaakt door ALF-gerelateerde componenten in het plasma, maar door de achtergrond van het plasma, mogelijk doordat stoffen normaal aanwezig in het kweekmedium ontbraken. De eliminatie van ammoniak en synthese functie van HepaRG cellen bleken relatief resistent tegen de toxische effect van ratten (ALF) plasma.

Vervolgens hebben we de effecten van ratten ALF plasma op de HepaRG-AMC-BAL (gekweekt zonder DMSO) beschreven in **Hoofdstuk 8**. We hebben de effecten van plasma van ratten met zowel mild als ernstig ALF onderzocht. De HepaRG-AMC-BALs werden getest vóór

blootstelling aan ALF plasma (controle groep), na 5 uur blootstelling aan plasma van ratten met milde (beginnende) ALF, of na 10 uur blootstelling aan plasma van ratten met vergevorderde ALF. De cellekkage nam maar marginaal toe na blootstelling aan zowel mild als ernstig ALF plasma. Echter, de transcript niveaus van bijna alle onderzochte genen namen af, met name de genen geassocieerd met detoxificatie en regulatie. Interessant genoeg bleven de hepatische functies vrijwel hetzelfde in de mild ALF groep, en namen zelf gemiddeld wat toe in de ernstig ALF groep. Deze studie leerde ons dat de HepaRG-AMC-BAL relatief resistent is tegen de toxische effecten van ALF plasma en dar er zelfs een tijdelijke (mogelijk compensatoire) toename is van de hepatische functionaliteit. Niettemin zal de functionaliteit wel afnemen bij nog langere blootstelling aan ALF plasma, omdat de transcript niveaus van vijwel alle onderzochte genen substantieel verlaagd werden door blootstelling aan ALF-plasma.

In **Hoofdstuk 9** beschrijven we het effect van de perfusiesnelheid van het medium door de HepaRG-AMC-BAL op de functionaliteit, en we beschrijven de massabalansen van verschillende substraten over de bioreactor. Hiertoe hebben we laboratorium versies van de HepaRG-AMC-BAL geperfundeerd met testmedium op 4 verschillende snelheden (0.3, 1.5, 5, and 10 mL/min) en het effect op verschillende hepatische functies (eliminatie van ammoniak, productie van ureum, lactaat consumptie en CYP3A4 activiteit) en cellekkage. Gemiddeld genomen was de hepatische functionaliteit het hoogst bij een perfusiesnelheid van 5 mL/min, te weten 2-20x zo hoog als bij 0.3 mL/min. Bij de hoogste snelheid van 10 mL/min was de functionaliteit ook hoog, maar nam de cellekkage toe. Dit laaste was waarschijnlijk het gevolg van directe fysieke schade aan de cellen door de hge snelheid ('shear stress') van de vloeistof. Al met al bleek 5 mL/min dus de meest optimale perfusiesnelheid van de laboratoriumversie van de HepaRG-AMC-BAL. Als we deze resultaten extrapoleren naar de BAL die voor patiënten gebruikt gaat worden, zou de optimale perfusiesnelheid circa 200-250 mL/min zijn.

Vervolgens berekenden we massa ballansen van ammoniak en lactaat over de HepaRG-AMC-BAL, met als doel om de BAL-functie te kunnen monitoren tijdens de behandeling van ALF patiënten. We lieten zien dat deze massa balansen tussen het inflow- en outflow-kweekmedium alleen betrouwbaar bepaald kunnen worden bij de laagste suboptimale snelheid van 0.3 mL/min in de setup waarin we getest hebben.

De HepaRG cellijn staat ook bekend als een goede tool voor het *in vitro* testen van het metabolisme en de toxiciteit van geneesmiddelen en andere chemische verbindingen. Echter, de detoxificatie functionaliteit van de HepaRG cellen (in monolayer kweken) is afhankelijk van de toevoeging van DMSO aan het kweekmedium, wat (zoals boven beschreven) celschade en onderdrukking van de allround hepatische functionaliteit veroorzaakt. In **Hoofdstuk 10** beschrijven we de detoxificatie functionaliteit van de HepaRG-AMC-BAL (gekweekt zonder DMSO) daarom uitgebreider. We lieten zien dat de HepaRG cellen in de HepaRG-AMC-BALs

zich organiseren als gepolariseerd lever-achtig weefsel met heterogene expressie van *CYP3A4*. We vonden substantieel metabolisme van de geteste geneesmiddelen en andere chemische verbindingen, variërend van 26% (UDP-glucuronosyltransferase 1A1), 47% (CYP3A4) to 240% (CYP2C9) het niveau van humane hepatocyten. Daarnaast produceerde de HepaRG-AMC-BAL galzouten met een snelheid van 43% die van humane hepatocyten, waarbij deze galzouten ook gehydroxyleerd, geconjugeerd en getransporteert werden. Deze resultaten laten zien dat DMSO overbodig is voor het verhogen van het detoxificatie functionaliteit, mits de HepaRG cellen in de AMC-BAL gekweekt worden. Dat maakt de AMC-BAL een zeer geschikt kweekomgeving voor de HepaRG cellen voor het testen van het metabolisme en de toxiciteit van geneesmiddelen en andere chemische verbindingen.

CONCLUSIES EN TOEKOMSTPERSPECTIEVEN

Het belangrijkste doel van dit proefschrift was het ontwikkelen van een geschikte humane biocomponent voor de AMC-BAL: een biocomponent die voldoende hepatische functionaliteit heeft, die expandeerbaar is zonder functionaliteit te verliezen, en die veilig is.

De studies in dit proefschrift laten zien dat de HepaRG cellijn een veelbelovende biocomponent is wat betreft functionaliteit. Namelijk, wanneer de HepaRG cellijn gekweekt wordt in de AMC-BAL zonder DMSO en in aanwezigheid van carbamoyl glutamate laat deze een hoge mate van hepatische funtionaliteit zien, en belangrijker, behandeling van ALF ratten met deze HepaRG-AMC-BAL verlengt de overlevingstijd substantieel. Verdere analyse laat zien dat de HepaRG-AMC-BAL relatief resistent is tegen de toxische effecten van ALF plasma. Daarnaast hebben we ook de optimale perfusiesnelheid voor het medium/plasma vastgesteld. Daarmee vormen deze studies de opmaat voor verdere klinische studies met de HepaRG-AMC-BAL.

Niettemin zijn er enkele belangrijke zaken die nog onderzocht moeten worden voor deze klinische studies plaats kunnen vinden. Allereerst zou de effectiviteit van de HepaRG-AMC-BAL in nog een ander diermodel, bij voorkeur een groot dier zoals een varken, moeten worden onderzocht met een bioreactor opgeschaald tot klinisch relevante omvang. In ogenschouw nemend dat er circa 30-40% functionele levermassa nodig is voor de homeostase, en dat de lever van ALF en ACLF patiënten over het algemeen nog wel (zij het weinig) wat restfunctie heeft, zou de HepaRG-AMC-BAL het equivalent van 15-20% van de levermassa moeten bevatten.

Daarnaast moeten ook de functionele stabiliteit en de veiligheid van de HepaRG-AMC-BAL nog onderzocht worden. Functionele stabiliteit kan worden onderzocht door middel van sequentiële functietests gedurende een langdurige kweekduur. Het testen van de veiligheid van de HepaRG-AMC-BAL omvat toxicologische testen en steriliteittesten van de AMC-BAL zowel met als zonder de HepaRG cellen, en van het extracorporele systeem. Daarnaast moeten de

HepaRG cellen uitvoerig onderzocht worden op eventuele tumorigeniciteit, aangezien de cellen afkomstig zijn van een hepatocellulair carcinoom. En zelfs als deze testen laten zien (zoals al is aangetoond in het verleden) dat de HepaRG cellen niet in staat zijn om tumoren te vormen in het lichaam, moeten alsnog filters in het extracorporele circuit worden opgenomen om eventuele losgeraakte cellen tegen te houden en te voorkomen dat ze in het lichaam komen.[8]

Idealiter zou BAL therapie ALF en ACLF patiënten niet moeten overbruggen naar transplantatie, maar leverfunctievervangende therapie moeten geven zolang als nodig is om de eigen lever te laten regenereren, om zo een transplantatie te voorkomen. Het rattenmodel van complete leverischemie waarin we de effectiviteit van de HepaRG-AMC-BAL hebben laten zien laat echter geen functionele levermassa bestaan en laat daarmee geen lever regeneratie toe. Daarom zou er een nieuwe dierstudie plaats moeten vinden waarin bijvoorbeeld een subtotale hepatectomie (het uitnemen van een deel van de lever) zou kunnen worden verricht, eventueel gecombineerd met een toxisch ALF model zoals een paracetamol overdosis of een galactosamine / lipopolysaccharide injectie.

Zoals in de introductie van dit proefschrift uiteengezet is, is de schadelijke rol van het aminozuur glutamine in de pathofysiologie van HE pas vrij recent ontdekt.[9] In het kort wordt ammoniak in het brein gefixeerd in glutamine en vervolgens naar de mitochondriën (energiefabrieken van de cel) van de astrocyten (type cel in het brein) getransporteerd. De intra-mitochondriale hydrolyse van glutamine door het enzym glutaminase die vervolgens plaatsvindt, maakt ammoniak weer vrij en verhoogt de ammoniakconcentratie lokaal sterk. Deze hoge toxische concentratie van ammoniak resulteert in oxidatieve stress (vorming van o.a. zuurstofradicalen), wat weer resulteert in het zwellen en disfunctioneren van de mitochondriën. De belangrjikste route van ammoniak eliminatie in de HepaRG-AMC-BAL blijkt de niet-geprefereerde fixatie in glutamine zijn – en niet de transformatie in ureum, dat veel minder toxisch is en door de nieren gemakkelijker kan worden uitgescheiden. Het gebruik van 'scavengers' van glutamine zoals fenylazijnzuur of benzoëzuur die glutamine kunnen binden waarna het gemakkelijk door de nieren wordt uitgescheiden kan de glutamineconcentratie in het plasma verlagen en zo bijdragen aan het detoxificeren van ammoniak.[10] Daarnaast is ook het verhogen van de ureumcyclus activiteit in de HepaRG-AMC-BAL, bijvoorbeeld door genen tot overexpressie te brengen die coderen voor de snelheids-bepalende enzymen van de ureumcyclus, een mogelijkheid die verder onderzocht kan worden.

In de zoektocht naar een geschikte biocomponent voor de AMC-BAL hebben we ons tot dusver gefocust op het vervangen van de functies van de hepatocyt, het belangrijkste en meest voorkomende celtype (circa 80% van alle levercellen) van de lever. Echter, andere levercellen vervullen belangrijke leverfuncties. De rol van de Kupffer-cellen, afweercellen die in het leverweefsel aanwezig zijn en veel pathogenen zoals endotoxines wegvangen, is in ALF en ACLF onduidelijk. Het stilleggen van de Kupffer-cellen kan de schade aan de lever namelijk zowel verergeren als tegengaan. Waarschijnlijk heeft dit te maken met het feit dat er verschillende typen

Kupffer-cellen bestaan (M1 en M2) die qua functie elkaars tegengestelde zijn: de ene hebben een anti-inflammatoire (ontstekingsremmende) werking en de andere een pro-inflammatoire werking.[11] Andere celtypen van de lever zoals de endotheelcellen (bloedvatcellen) en de hepatische stercellen kunnen de hepatocytische functionaliteit stabiliseren en ook verhogen.[12] Daarom zijn co-kweken van HepaRG cellen met deze endotheelcellen en/of hepatische stercellen in de AMC-BAL een interessante optie die verder onderzocht kan worden. Daarnaast is een co-kweek van HepaRG cellen met mesenchymale stamcellen, multi-potente stromale cellen die o.a. een cytokine-gemedieerd immunosuppressief (afweeronderdrukkend) effect kunnen uitoefenen, een interessante optie om verder te onderzoeken.[13]
Wordt vervolgd.

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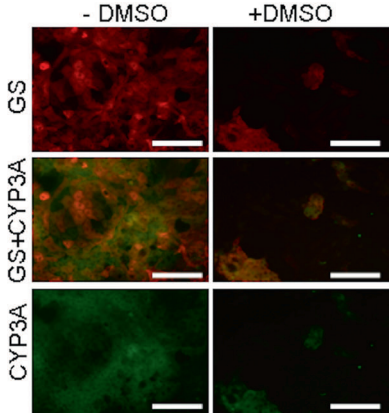
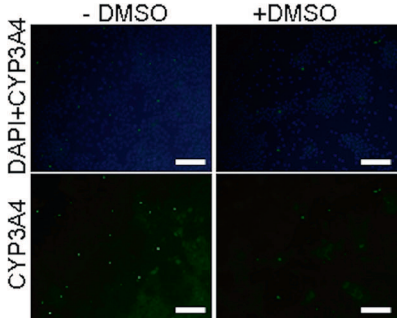
APPENDIX 1

Color images

Chapter 1. Figure 1. Photograph of the AMC-BAL.



Chapter 4. Figure 2. Immunofluorescence double stainings for CYP3A4 and GS. Bars represent 100 μm.



Chapter 4. Figure 3. Immunofluorescence staining for CYP3A4 with DAPI counterstaining. Bars represent 100 μm.

Chapter 2. Table 1. Overview of proliferative human cell sources applied in bioartificial livers.

Colors indicate the potential for BAL application as indicated by in vitro functionality or efficacy in animal studies:
white: no data available.
red: in vitro functionality < 5% of primary hepatocytes, or not effective in animal study;
yellow: inconclusive
green: in vitro functionality > 10% of primary hepatocytes, or proven efficacy in animal study

Biocomponent	Origin	Bioreactor	Tumorgenicity	Growth potential	In vitro functionality	
					Synthesis	Drug-metabolizing activity
	Hepatoma cells					
HepG2	Hepatocellular carcinoma, 15 year old male.	Diverse devices	Pos in immunocomprimised mice *	Immortal	Pos Alb, AFP, ApoA1, fibrinogen	Neg/low in phase I and phase II detoxification
C3A	Clonal derivative of HepG2, selected for strong contact inhibition, high production of albumin and AFP and growth in glucose deficient medium	Diverse devices, most often in ELAD	Neg in immunocomprimised mice	Immortal	Pos Alb, AFP, transferrin	Detectable 7-ethoxycoumarin metabolism, level inconclusive
Huh 7	Hepatoma	Polyurethane foam/sheroid	Pos in immunocomprimised mice	Immortal	Pos Alb	
FlC	Hepatoma	Radial flow		Immortal		
FLC-4	Hepatoma	Radial flow		Immortal	Pos Alb, coagulation factors	Detectable CYP mRNAs, level inconclusive
FLC-5	Hepatoma	Radial flow		Immortal	Pos Alb, coagulation factors	Detectable 6b-hydroxylation, level inconclusive
FLC-7	Hepatoma	Radial flow		Immortal	Pos Alb, coagulation factors	Detectable CYP mRNAs, level inconclusive
HepaRG	Hepatoma	AMC-BAL and multi-compartment hollow fiber capillary membrane	Neg in immunocompromised mice	Immortal	Pos ApoA1	Pos CYP3A4, 6-b hydroxylation testosterone

			Animal studies	Clinical studies	Reference
Urea production**	NH_3 elimination	Carbohydrate metabolism			
Pos, only due to Arg II activity	Inconsistency between reports	Lactate production and glucose consumption	One animal study (n=3) in rat with acetaminophen intoxication using a bioreactor based on alginate incapsulated cells: no effect on survival measured, no effect found on ammonia & bilirubin levels		Nyberg et al. Ann Surg.1994 Yamashita et al. Cell Transplant. 2001 Fukada et al. Cell Transplant. 2003 Rahman et al. Artif Org 2004 Hongo et al. J Biosci Bioeng. 2005 Shimada et al. Hepatogastroenterol. 2007 Coward et al. Art Org. 2009 Nibourg et al. Liver Transplant. 2010
Pos, only due to Arg II activity	Detectable, level inconclusive	Glucose consumption	Only in ELAD. Two inconclusive animal studies (n=3) in anhepatic dogs and dogs with acetaminphen intoxication.	Two clinical phase I studies showing safety. Two randomized controlled clinical trials; the first one not showing positive effects and the second one with unpublished results.	Sussman et al. Hepatol. 1992 Kelly et al. Artif Org. 1992 Sussman et al. Artif Org. 1994 Ellis et al. Hepatol. 1996 David et al. Biotechnol Prog. 2004 Kinasiewicz et al. Transplant Proc. 2007 Kinasiewicz et al. Artif Org. 2008 Kinasiewicz et al. Artif Cells Blood Substit. 2008 Kinasiewicz et al. Int J Artif Org. 2008 Carraro et al. Biomed Microdev. 2008 Harm et al. Int J Artif Org. 2009 Chen et al. Tissue Eng Part A. 2010 Gautier et al. Eur Cells Mat. 2011
					Yamashita et al. Cell Transplant. 2001
					Iwahori et al. Transplant Proc. 2005
No CPS and OTC expression			One animal study (n=2-3) in pigs with α-amanitin and lipopolysaccharide intoxication. Inconclusive results.		Kosuge et al. Liver Int. 2007 Kanai et al. Artif Org. 2007
No CPS and OTC expression					Iwahori et al. Hepatol. 2003 Kosuge et al. Liver Int. 2007
No CPS and OTC expression		Glucose consumption			Kawada et al. In Vitro Cell Dev Biol Animal. 1998 Kosuge et al. Liver Int. 2007
		Lactate consumption, stable glucose levels	One animal study (n=5, 6) in rats with total liver ischemia: positive effect on survival time, HE, bleeding, creatinine and ammonia levels		Darnell et al. Drug Metab Dispos. 2011 Nibourg et al. Unpublished data.

Table 1. Continued

	In vitro immortalized cells					
HepZ	Hepatocyte cell line immortalized through E2F and cyclin D1 overexpression and suppression of pRB and P53	Based on microporous gelatin microcarriers		Immortal		Detectable lidocaine metabolism, level inconclusive
OUMS-29	Fetal hepatocyte cell line immortalized through expression SV40 large and small T antigen	Radial flow		Immortal	Pos Alb	
cBAL111	Fetal hepatocytes immortalized through hTERT overexpression	AMC-BAL		Immortal	Alb level only 6%	
Kobayashi revers imm hTERT PHH	Hepatocytes reversibly immortalized through hTERT overexpression	Hollow fiber		Immortal		
	Fetal cells and stem cells					
Fetal hepatocytes	Fetal liver	AMC-BAL and hollow-fiber		Limited	Pos Alb	Lidocaine metabolism
Small hepatocytes	Liver	Rotary cell culture with cytodex microcarriers		Limited	Relatively low Alb, and substantial decline in time	CYP3A4 protein expression and metabolism of methadone however, no metabolism of morphine and susceptible to toxins
Liver stem cells	Liver	Rotary system		?	Pos Alb	Metabolism 7-ethoxy-4-trifluoromethylcoumarin
Mesenchymal stem cells	Differentiated bone-marrow cells	Flat plate or with 3D nanofibrous scaffold		?	Transferrin, Alb detectable, level inconclusive	Detectable CYP3A4 mRNA, level inconclusive
Differentiated human embryonic stem cells	Blastocyst	Multi-compartment hollow fiber capillary membrane		?	Alb level only <1%	Detectable CYP3A4 and CYP3A7 mRNA, level inconclusive

	Production	Lactate production converting to consumption and glucose consumption			Werner et al. Ann New York Acad Sci. 1999 Werner et al. Biotechnol Bioeng. 2000
					Akiyama et al. Int J Mol Med. 2004
		Lactate production and glucose consumption	One animal study (n=4) in rats with total liver ischemia. No positive effect on survival time.		Poyck et al. J Hepatol. 2008
			Two animal studies. The first study in monkeys with D-galactosamine intoxication with inconclusive results. The second study in pigs with ALF, ongoing.		Kobayashi et al. J Artif Org. 2003 Kobayashi et al. J Hepatobil Pancreat Surg. 2009
	Production	Lactate production and glucose production converting into consumption			Poyck et al. Liver Transpl. 2007 Ring et al. Tissue Eng Part C. 2010 Schmelzer et al. Tissue Eng Part A. 2010
Pos, but substantial decline in time		Lactate production and glucose consumption, but both parameters decline substantially in time			Pavlic et al. Alcohol Clin Exp Res. 2007 Wurm et al. Tissue Eng Part A. 2009 Wurm et al. Tissue Eng Part A. 2010
		Glucose consumption			Fonsato et al. Tissue Eng Part C. 2009
Detectable, level inconclusive			One animal study (n=7) in rats with D-galactosamine intoxication. Positive effects on survival and on leakage of AST and ALT.		Parekkadan et al. PLoS One. 2007 Kazemnejad et al. J Gastroenterol Hepatol. 2009
Detectable, level inconclusive		Lactate production and glucose consumption			Miki et al. Tissue Eng Part C. 2011

Table 1. Continued

	Genetically modified cells					
HepG2-GS	HepG2 with overexpression GS	Radial flow		Immortal		
HepG2-GS-Cyp3A4	HepG2 with overexpression GS and Cyp3A4	Radial flow		Immortal		Detectable diazepam metabolism, level inconclusive
HepG2-DT	HepG2 with overexpression OTC and ArgI	Fluidized bed based on alginate incapsulated cells		Immortal	Pos Alb	
cBAL119	HepG2 with overexpression PXR	AMC-BAL		Immortal	Pos ApoA1	Enhanced 6-b hydroxylation testosterone compared to HepG2, but still low
OUMS-29/H-11	OUMS-29 with overexpression HNF4	Radial flow		Immortal	Pos Alb	CYP3A4 protein present, level inconclusive. Enhanced 6-b hydroxylation testosterone in late culture phase, but still low
TTNT with AdIL-1Ra	Hepatocytes reversibly immortalized through hTERT overexpression, and further expressing IL-1 recepor antagonist	Flat-plate		Immortal		
	Combinations of cells					
FLC-5, M1, and A7	Human hepatoma cell line with murine cell lines (endothelial and stellate)	Radial flow			Alb production FLC-5 decreased by co-culturing	
HepG2-GS-CYP3A4 and PCTL-MDR	Modified hepatoma cell line with rabbit renal cell line transduced with MDR	Compartment bioreactor				
HLSC, stellate cells	Human liver stem cells with human stellate cells	Perfusion bioreactor wiht porous scaffold			Pos Alb	

* although the ATCC datasheet states that HepG2 does not form tumours in SCID mice, several investigators use HepG2 cells to induce tumour formation in SCID mice {Sun, 2004 459 /id;Lu, 2003 460 /id}.

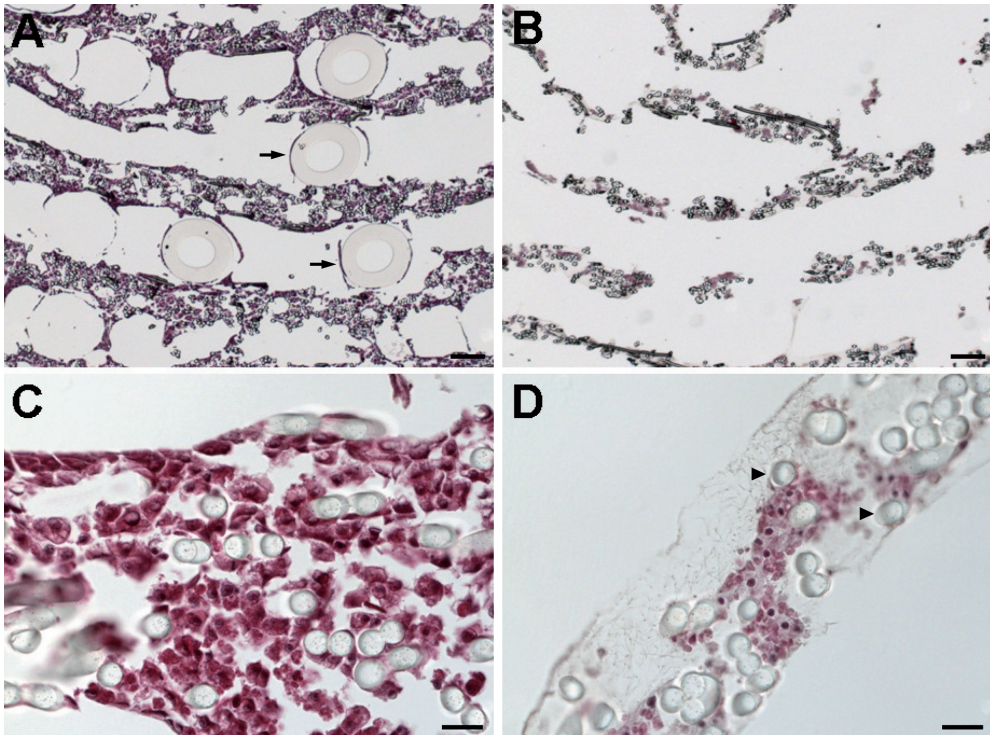
** Ureagenesis can be positive without a functional urea cycle.

Abbreviations: AFP, alpha fetoprotein; Alb, albumin; ApoA1, apolipoprotein A-1; Arg, arginase; ALF, acute liver failure; AMC-BAL, Academic Medical Center-BAL; CYP, cytochrome P450; ELAD extracorporeal liver assist device; elim, elimination; GS, glutamine synthetase; HE, hepatic encephalopathy;

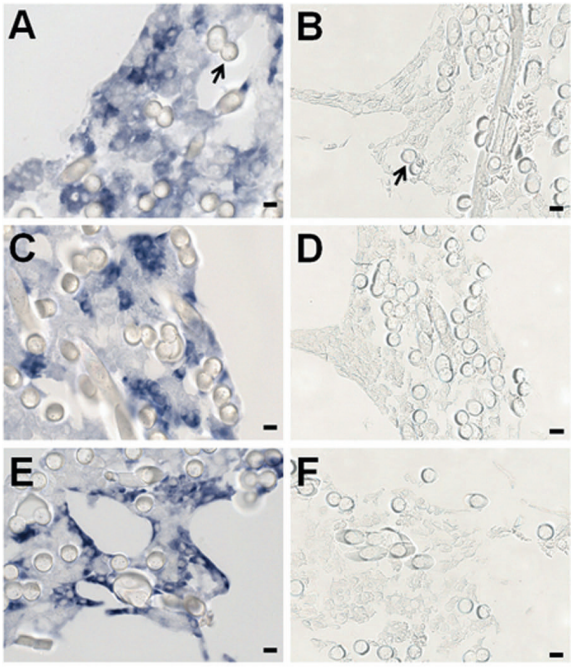
HNF4, hepatocyte nuclear factor 4; hTERT, human telomere reverse transcriptase; neg, negative; IL-1, interleukine 1; OTC, ornithine transcarbamoylase; pos, positive; pRb, Retinoblastoma protein; prod, production; PH, primary hepatocyte; PXR, pregnane X receptor; revers imm PHH, reversibly immortalized primary human hepatocytes; SV40, simian virus 40.

			Two animal studies. The first study in pigs with total liver ischemia (n=8-9): positive effect on survival time. The second study in dogs with total liver ischemia (n=5-7): no effect on survival time		Enosawa et al. Cell Transplant. 2000 Enosawa et al. Cell Transplant. 2001 Enosawa et al. Transplant Proc. 2001 Enosawa et al. Cell Transplant. 2006
			One animal study (n=7-8) in dogs with total liver ischemia: pos effect on survival time and diazepam metabolism		Wang et al. Artif Organs. 2005
					Coward et al. Artif Organs. 2009
		Lactate production and glucose consumption			Nibourg et al. Liver Transplant. 2010
					Akiyama et al. Int J Mol Med. 2004
			One animal study (n=9) in rats with D-galactosamine intoxication. No positive effects on survival. Significant reduction IL-6 levels.		Shinoda et al. J Surg Res. 2007
No OTC expression					Saito et al. World J Gastroenterol. 2006
		Lactate production			Endo et al. Art Org. 2002 Takahashi et al. Cell Transplant. 2006
					Carraro et al. Tissue Eng Part C. 2011

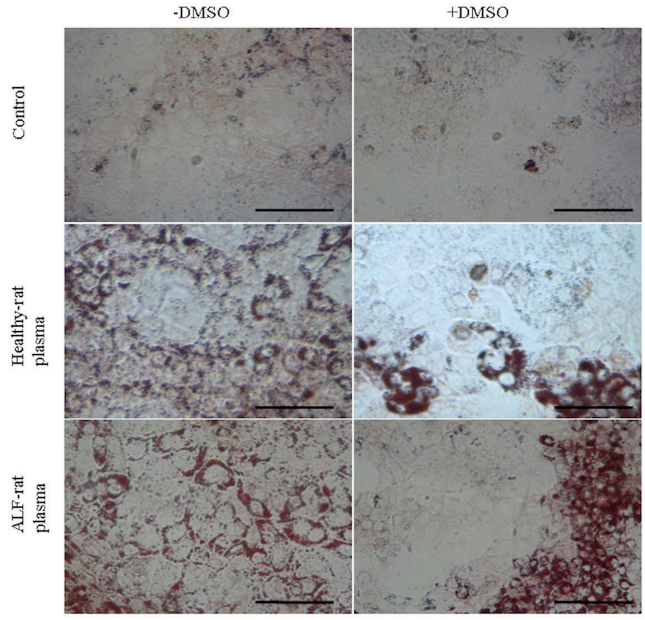
Colors indicate the potential for BAL application as indicated by in vitro functionality or efficacy in animal studies. White: no data available. Red: in vitro functionality < 5% of primary hepatocytes, or not effective in animal study. Yellow: inconclusive. Green: in vitro functionality > 10% of primary hepatocytes, or proven efficacy in animal study. * Although the ATCC datasheet states that HepG2 does not form tumours in SCID mice, several investigators use HepG2 cells to induce tumour formation in SCID mice.[101, 102] ** Ureagenesis can be positive without a functional urea cycle. Abbreviations: AFP, alpha fetoprotein; Alb, albumin; ApoA1, apolipoprotein A-1; Arg, arginase; ALF, acute liver failure; AMC-BAL, Academic Medical Center-BAL; CYP, cytochrome P450; ELAD extracorporeal liver assist device; elim, elimination; GS, glutamine synthetase; HE, hepatic encephalopathy; HNF4, hepatocyte nuclear factor 4; hTERT, human telomere reverse transcriptase; neg, negative; IL-1, interleukine 1; OTC, ornithine transcarbamoylase; pos, positive; pRb, Retinoblastoma protein; prod, production; PH, primary hepatocyte; PXR, pregnane X receptor; revers imm PHH, reversibly immortalized primary human hepatocytes; SV40, simian virus 40.



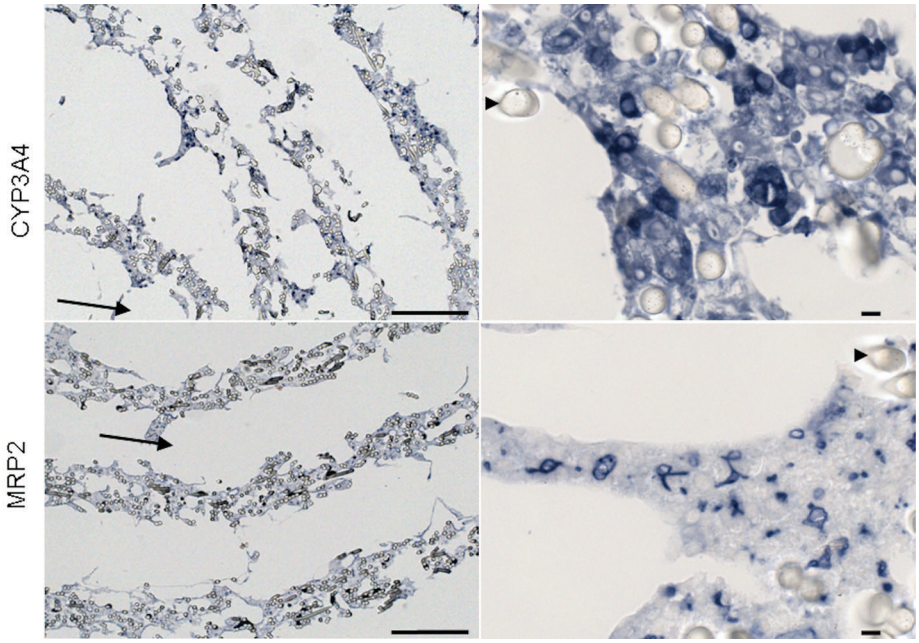
Chapter 5. Figure 3. HA stainings of cross sections of -DMSO (A, C) and +DMSO HepaRG-AMC-BALs (B, D). Full transverse sections (A, B) show the spirally wound matrix layers with the gas capillaries (arrows) positioned in between. Details of the matrix (C, D) show the polyester matrix fibers (arrowheads) with HepaRG cells, and the web-shaped extracellular matrix in acellular areas in the matrix (D). Bars: 200 μm (A, B) and 20 μm (C, D).



Chapter 6. Figure 1. Immunostainings of cross-sections of d14 HepaRG-AMC-BALs. Proteins stained were GS (A) with its negative control (B), CPS (C) with its negative control (D), and ALB (E) with its negative control (F). Fibers of the AMC-BAL's matrix are indicated with arrows. Bars: 10 μm.



Chapter 7. Figure 2. The effect of healthy and ALF plasma on the lipid accumulation of -DMSO and +DMSO HepaRG monolayers. Negative controls are included. Bars represent 100 μm.



Chapter 10. Figure 1. CYP3A4 and MRP2 stainings of cross sections of HepaRG-AMC-BALs at 14 days of culture at two different magnifications. The full transverse sections (left, bars: 500 μm) show the spirally wound matrix layers between gas capillaries (arrows show spaces left after washing off capillaries by experimental procedure) in between. Details of the matrix (right, bars: 10 μm) show the matrix fibers (arrowheads) with HepaRG cells with heterogeneous CYP3A4 distribution and canalicular MRP2 localization.

APPENDIX 2

Abbreviations

6β-OHT	6β-hydroxytestosterone
AFP	alpha-fetoprotein
ALB	albumin
ALF	acute liver failure / acuut leverfalen
AMC	Academic Medical Center / Academisch Medisch Centrum
AMC-BAL	Academic Medical Center bioartificial liver
ANOVA	one-way analysis of variance
ACLF	acute-on-chronic liver failure / acuut-op-chronisch leverfalen
ApoA-1	apolipoprotein A-1
ARG	arginase
ARG1	arginase 1
ARG2	arginase 2
ASL	argininosuccinate lyase
ASS	argininosuccinate synthetase
AST	aspartate aminotransferase
BAL	bioartificial liver / bioartificiële lever
CA	cholic acid
CAR	constitutive androstane receptor
CG	carbamoyl-glutamate
CLI	complete liver ischemia.
CPS	carbamoylphosphate synthetase
CYP	cytochrome P450
CYP3A	cytochrome p450 3A
DAPI	4,6-diaminidino-2-phenylindole
DFB	3-[(3,4-difluorobenzyl)oxy]-5,5-dimethyl-4-[4-(methylsulfonyl) phenyl]furan-2(5*H*)-one
DFH	3-hydroxy-5,5-dimethyl-4-[4-(methylsulfonyl)phenyl]furan-2(5*H*)-one
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide / dimethylsulfoxide
ELAD	extracorporeal liver assist device
ELF	ernstig leverfalen
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FLC	fetal liver cell
g-CA	glycine-conjugated cholic acid
g-CDCA	glycine-conjugated chenodeoxycholic acid
GS	glutamine synthetase
HE	hepatic encephalopathy / hepatische encephalopathie

HGF	hepatocyte growth factor
HLSC	Human liver stem cells
HNF	hepatic nuclear factor
HNF4A	hepatic nuclear factor 4α
HNF4α	hepatic nuclear factor 4α
HPLC	high performance liquid chromatography
HSV-tk	herpes simplex virus type 1 thymidine kinase
hTERT	human telomerase reverse transcriptase
IgG	immunoglobulin G
IL-1Ra	interleukin 1 receptor antagonist
LDH	lactate dehydrogenase
LT	liver transplantation
MDR	multidrug resistance protein
MOF	multi-organ failure
MRP2	multidrug resistance-associated protein 2
MSC	mesenchymal stem cell
ND	not determined
NI	not identified
ns	not significant
OTC	ornithine transcarbamoylase
PBS	phosphate-buffered saline
PGK	phosphoglycerate kinase
PH	primary hepatocyte
PHHs	primary human hepatocytes
PPH	primary porcine hepatocyte
pRb	retinoblastoma protein
PRH	primary rat hepatocyte
PXR	pregnane X receptor
RT-PCR	reverse transcriptase polymerase chain reaction
SH	small hepatocyte
SLF	severe liver failure
t-CA	taurine-conjugated cholic acid
t-CDCA	taurine-conjugated chenodeoxycholic acid
TF	transferrin
UDCA	ursodeoxycholic acid
UGT	UDP-glucuronosyltransferases

APPENDIX 3

List of publications

Hoekstra R, **Nibourg GA**, van der Hoeven TV et al. The HepaRG cell line is suitable for bioartificial liver application. Int J Biochem Cell Biol 2011;43(10):1483-1489.

Nibourg GA, Huisman MT, van der Hoeven TV, van Gulik TM, Chamuleau RA, Hoekstra R. Stable overexpression of Pregnane X receptor in HepG2 cells increases its potential for bioartificial liver application. Liver Transpl 2010;16(9):1075-1085.

Nibourg GA, Chamuleau RA, van der Hoeven TV et al. Liver Progenitor Cell Line HepaRG Differentiated in a Bioartificial Liver Effectively Supplies Liver Support to Rats with Acute Liver Failure. PLoS One 2012;7(6):e38778.

Nibourg GA, Chamuleau RA, van Gulik TM, Hoekstra R. Proliferative human cell sources applied as biocomponent in bioartificial livers: a review. Expert Opin Biol Ther 2012;12(7):905-921.

Nibourg GA, Boer JD, van der Hoeven TV et al. Perfusion flow rate substantially contributes to the performance of the HepaRG-AMC-bioartificial liver. Biotechnol Bioeng 2012.

Hoekstra LT, de Graaf W, **Nibourg GA** et al. Physiological and Biochemical Basis of Clinical Liver Function Tests: A Review. Ann Surg 2012.

Hoekstra R, **Nibourg GA**, van der Hoeven TV et al. The effect of rat acute-liver-failure plasma on HepaRG cells. Int J Artif Organs. *In press.*

APPENDIX 4

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Dr. R.A.F.M. Chamuleau, mijn co-promotor: Beste Rob, ik heb je de afgelopen vier jaar niet alleen leren kennen als een kundige mentor met een schat aan ervaring, maar zeker ook als een zeer hartelijk persoon. Onder jouw bezielende leiding staat de BAL-groep al jaren als een huis. De BAL vergaderingen waren altijd kritisch, open en constructief, en de sfeer was altijd goed. Ik vond het een ideale omgeving om me als onderzoeker te kunnen ontwikkelen en heb veel van je geleerd. Veel dank hiervoor!

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De BAL-groep als geheel. Beste Rob, Ruurdtje en Tessa, de afgelopen vier jaar heb ik bovenal ervaren als heel mooie en leerzame jaren. Maar ook als bewogen jaren, want we hebben onze fair share van pieken en dalen wel gehad de afgelopen vier jaar. Toen ik begon in 2007, stonden we eigenlijk op nul, want van de cBAL111 cellen was net gebleken dat ze 'het niet deden'. Ook de cBAL119 cellen (Hoofdstuk 3) bleken niet de humane biocomponent waar we naar zochten. Maar toen kwamen de HepaRG cellen (na maanden aandringen) uit Frankrijk en die zagen er na de eerste monolayer testen meteen veelbelovend uit. Ook in de BAL deden ze het *in vitro* boven verwachting goed, dus dat er opnieuw rattenproeven gingen komen stond al snel vast.

Maar dat bleek geen sinecure... Twee jaar heeft het geduurd voordat ik eindelijk die felbegeerde overlevingscurve (Hoofdstuk 5, figuur 5A) kon laten zien. Bijna wekelijks experimenten, vaak tot in de nachtelijke uren, steeds weer weekenden terugkomen om de kostbare BAL-kweken te checken, een niet meer te tellen aantal dozen kweekmedium, het zoveelste opnieuw aangepaste kweekschema voor de monolayer expansiekweken, soms de wanhoop nabij, weer sleutelen aan het model (twee parallelle plasmaferese-units in plaats van één), meer discussies, schimmelinfecties, weer de literatuur in, nog weer een andere aanpassing aan het model (toch weer een 'high flow BAL-circuit' erin), et cetera. En toen was er ineens die rare variatie binnen de groepen. Ik zal het moment nooit vergeten dat we erachter kwamen dat de wisselende intestinale bacteriële urease activiteit van de ratten de boosdoener was! Het eureka-moment van de afgelopen vier jaar wat mij betreft. Met de invoering van de Rifaximin toedieningen vóór de BAL behandeling, daalde de variatie binnen de groepen aanzienlijk. De sms'jes met de overlevingstijden die ik jullie steeds na elk experiment stuurde, verplaatsten zich daarna ook van halverwege de avond naar midden in de nacht als er een HepaRG-BAL 'aanhing'. En niet veel later was de klus geklaard: de HepaRG cellen doen het! Dat we die EU subsidie binnengehaald hebben en dat die klinische studie er nu echt gaat komen vind ik eigenlijk de mooiste beloning van vier jaar hard werken. Met Martien en Erik als nieuwe aanwinsten wens ik jullie allemaal heel veel succes in deze mooie tijden die aanbreken. Op naar de kliniek met de HepaRG-AMC-BAL!

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