Hemoglobin Regulates the Metabolic, Synthetic, Detoxification, and Biotransformation Functions of Hepatoma Cells Cultured in a Hollow Fiber Bioreactor

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Hepatic hollow fiber (HF) bioreactors constitute one type of extracorporeal bioartificial liver assist device (BLAD). Ideally, cultured hepatocytes in a BLAD should closely mimic the *in vivo* oxygenation environment of the liver sinusoid to yield a device with optimal performance. However, most BLADs, including hepatic HF bioreactors, suffer from O₂ limited transport toward cultured hepatocytes, which reduces their performance. We hypothesize that supplementation of hemoglobin-based O₂ carriers into the circulating cell culture medium of hepatic HF bioreactors is a feasible and effective strategy to improve bioreactor oxygenation and performance. We examined the effect of bovine hemoglobin (BvHb) supplementation (15 g/L) in the circulating cell culture medium of hepatic HF bioreactors on hepatocyte proliferation, metabolism, and varied liver functions, including biosynthesis, detoxification, and biotransformation. It was observed that BvHb supplementation supported the maintenance of a higher cell mass in the extracapillary space, improved hepatocyte metabolic efficiency (i.e., hepatocytes consumed much less glucose), improved hepatocyte capacity for drug metabolism, and conserved both albumin synthesis and ammonia detoxification functions compared to controls (no BvHb supplementation) under the same experimental conditions.

Introduction

OXYGEN TRANSPORT STILL remains one of the major limiting factors in large-scale mammalian cell culture.^{1–4} It is well known that O₂ is sparingly soluble in the aqueous medium (~0.2 mmol/L at 1 atm air, 37°C). As a result, the cell culture medium in most cell culture systems need to be oxygenated to supraphysiological levels (>160 mm Hg) to deliver enough O₂ to cultured cells, which results in a portion of the cultured cells being exposed to hyperoxic conditions. Prolonged exposure to these conditions will induce the formation of reactive O₂ species (ROS), which will eventually kill cells.^{5,6}

Many methods have been proposed and utilized to improve O₂ delivery in large scale cell culture systems, including redesign of the bioreactor by computational modeling to alleviate mass transfer limitations, and supplementation of O₂ carriers (hemoglobin-based and perfluorocarbon-based) into the cell culture medium to increase the solubility of O₂ in the aqueous medium. Computational fluid dynamics has long been used to design and to optimize bioreactors by simultaneously modeling momentum and mass transfer.^{7,8} The use of O₂ carriers in the cell culture system, on the other hand, provides a biomimetic approach to recapitulate the *in vivo* oxygenation environment for many tissue engineering applications.^{9–11}

In particular, hepatic hollow fiber (HF) bioreactors that constitute one type of bioartificial liver assist device (BLAD) suffer from O₂ limited transport mainly due to the low solubility of O₂ in the cell culture medium, long diffusion path-lengths, and high demand for O₂ by the hepatocytes cultured in the extracapillary space (ECS).^{12–14} These devices are expected to bridge patients suffering from acute liver failure toward native liver regeneration or orthotopic liver transplantation by providing sufficient global liver functions.^{15,16}

In vivo, blood enters the periportal region of liver sinusoid via the hepatic artery and portal vein at a mean pO₂ of ~65 mm Hg and then leaves the sinusoid in the perivenous region via the central vein at a pO₂ of ~25–35 mm Hg, forming an O₂ gradient along the length of the liver sinusoid. The O₂ gradient along the length of the liver sinusoid is one of the factors controlling the liver's varied metabolic and synthetic functions.¹⁷ For instance, hepatocytes exhibiting gluconeogenesis functionality seem to be localized in the periportal region (where the local pO₂ is highest in the sinusoid), while cytochrome P450 activity is predominantly found in the perivenous region (where the local pO₂ is lowest in the sinusoid).¹⁸ The regulation of liver zonation is controlled at the transcriptional level, and it seems to be tightly coupled to the O₂ gradient via O₂-sensitive transcription factors.¹⁷ Therefore, provision of appropriate oxygenation to

hepatocytes cultured within a BLAD at similar levels, including reproducing the O_2 gradient observed physiologically, should result in a better functioning device.¹⁸

Supplementing hemoglobin-based O_2 carriers into the cell culture medium provides an effective and feasible strategy to improve O_2 transport in a hepatic HF bioreactor and to dually recapitulate the *in vivo* O_2 gradients. This has been demonstrated by extensive simulation work conducted in our lab.^{19–22} Recent experimental studies also showed that supplementation of bovine red blood cells (bRBCs) into the circulating cell culture medium improved O_2 transport to cultured C3A cells in a HF bioreactor.^{23,24} In the presence of bRBCs, the molar ratio of lactate production to glucose consumption of hepatoma cells was remarkably reduced, while albumin synthesis increased substantially, which indicates highly efficient cellular metabolism and synthetic function.

Despite promising results from the aforementioned theoretical and limited experimental studies, a thorough examination of the effect of hemoglobin-based O_2 carrier supplementation of the circulating cell culture medium of a hepatic HF bioreactor on cell proliferation, metabolism, and varied liver functions is obviously needed to validate this approach. In this study, experiments and mathematical analysis were performed to examine and analyze the effect of bovine hemoglobin (BvHb) supplementation in the circulating cell culture medium of a hepatic HF bioreactor on hepatocyte proliferation, metabolism, and varied liver functions, including biosynthesis, detoxification, and biotransformation.

Materials and Methods

C3A cell line

C3A cells (human hepatoma cell line; ATCC) were grown until confluent in T flasks in RPMI 1640 medium supplemented with 10% fetal bovine serum and 0.5% penicillin-streptomycin. Cells were harvested with 0.25% trypsin and counted on a hemacytometer, and viability was determined via Trypan blue exclusion before being inoculated into bioreactors.

BvHb purification

BvHb was purified from fresh bRBCs (Quad Five) via a three-stage HF filtration process as previously described in the literature.²⁵ Specifically, bRBCs were initially washed three times and subsequently lysed on ice. The RBC lysate was then filtered through a glass column packed with glass wool to remove the majority of cell debris. Clarified bRBC lysate was then passed through 50 nm and 500 kDa HF cartridges (Spectrum Labs) to remove additional cell debris and impurity proteins. Purified BvHb was then collected and concentrated on a 100 kDa HF cartridge (Spectrum Labs). The BvHb solution was sterile filtered before being introduced into the circulating cell culture medium of the experimental bioreactor systems.

Hepatic HF bioreactor system

The CellMax® Quad System (Spectrum Lab) with HF bioreactor module #410–011 was employed in this study. The schematic of this system is shown in Figure 1. The entire HF

FIG. 1. Schematic of the HF bioreactor system and O_2 transport model. (A) Schematic of the entire HF bioreactor system. (B) Close-up view of the HF cartridge. (C) Schematic of a single HF inside the cartridge. HF, hollow fiber. Color images available online at www.liebertonline.com/ten.

bioreactor system (Fig. 1A) was kept inside a humidified cell/tissue incubator (Innova CO-170 CO_2 incubator; New Brunswick Scientific Co.) at either 10% O_2 or 19% O_2 . Nineteen percent O_2 was chosen as it is close to the environmental pO_2 level in air at an atmospheric pressure of 1 atm. Ten percent O_2 corresponds to a pO_2 level of 80 mm Hg and was chosen to help recreate the *in vivo* O_2 gradient in the HF bioreactor as observed in the liver sinusoid (the inlet pO_2 at the sinusoid inlet is ~ 65 mm Hg). In the HF bioreactor system, the cell culture medium was continuously pumped from the reservoir bottle to the HF cartridge (i.e., through the lumen of individual HFs), delivering nutrients and O_2 to hepatocytes maintained within the ECS of the HF bioreactor while carrying away metabolic by-products. The overall medium flow rate was fixed at 1.5 mL/min throughout this study. There are 2024 HFs in the bioreactor cartridge and each HF has an internal radius of 0.01 cm. The overall volumetric flow rate of 1.5 mL/min gives a maximum velocity of ~ 0.4 mm/s, which is 60% higher than the *in vivo* hepatic erythrocyte flow velocity in rats of ~ 0.25 mm/s.²⁶ Three-way stopcocks were installed at the inlet and outlet of the HF cartridge, which enabled easy in-line sampling of the cell culture medium. The silicon tubing connecting the HF bioreactor to the medium reservoir bottle and pump is gas permeable, allowing the cell culture medium to be oxygenated at the ambient O_2 level inside the incubator before entering the HF cartridge (Fig. 1B). The individual HFs (Fig. 1C) in the cartridge consist of a cellulosic membrane with a 35 kDa molecular weight cutoff, so that BvHb (68 kDa) is confined to the lumen of the HF and cannot enter the ECS.

Hepatocyte culture in 3D HF bioreactor

About 10^7 C3A cells were inoculated into each HF cartridge via the ECS ports after 3 days of system priming (i.e.,

circulation of the cell culture medium in the HF cartridge without any cells present). The cell culture medium consisted of RPMI 1640 medium supplemented with 10% fetal bovine serum and 0.5% penicillin–streptomycin. After 24 h of culture (day 1), the cell culture medium in the reservoir of experimental HF bioreactors was replaced with a fresh medium supplemented with BvHb (15 g/L), while the cell culture medium in the hepatocyte-containing ECS of each experimental bioreactor was replaced with a fresh medium without BvHb. The cell culture medium in the reservoir bottle and ECS were then replaced every 48 h as previously described. The control bioreactors, however, had no BvHb supplementation in both the reservoir bottle and ECS at each medium change throughout the duration of the study. In addition, ammonium chloride (Fisher Scientific) was added to the reservoir at a concentration of 5 mM at each medium change in both control and BvHb systems. On day 13, lidocaine (Sigma-Aldrich) and 4-MU (4-methylumbelliferone; Sigma-Aldrich) were simultaneously administered to cultured hepatocytes at concentrations of 10 μ g/mL and 60 μ M, respectively. Drug metabolism in the liver involves two major sets of reactions: phase I biotransformation (cytochrome P450 activity) and phase II biotransformation (conjugation reactions). The metabolism of lidocaine is an indicator of phase I activity. This drug is mainly used as a local anesthetic, and is predominantly metabolized to monoethylglycinexylidide and 3-hydroxylidocaine in the human liver. 4-MU is generally used to evaluate the phase II activity as it undergoes both glucuronidation and sulfation *in vivo*.

After 24 h (day 14), cells were harvested from the ECS of each bioreactor following Accutase (Innovative Cell Technologies) treatment, and counted on a hemacytometer. Viability was determined via Trypan blue exclusion. O_2 tension (pO_2), CO_2 tension (pCO_2), and pH were measured every 48 h via a blood gas analyzer (RapidLab™ 248 pH/Blood Gas Analyzer; Chiron Diagnostics) at the inlet and outlet of each bioreactor.

Both control (no BvHb) and experimental bioreactors (containing BvHb) were run in duplicate. Two HF inlet pO_2 levels were studied in this work by incubating the bioreactor systems at either 10% O_2 or 19% O_2 inside the cell/tissue incubator. Additional HF bioreactor experiments were performed according to a similar medium exchange schedule except without the administration of ammonium chloride and drugs (lidocaine and 4-MU), and will be referred as the no drug group as compared to the drug group as previously described.

Biochemical analysis

Aliquots taken from the spent medium in the medium reservoir bottle at each medium exchange were assayed for methemoglobin (metHb) concentration (for experimental systems, via the cyanomethemoglobin method²⁷), glucose concentration and lactate concentration (YSI 2700 Select Biochemistry Analyzer; YSI Incorporated), and ammonia concentration (via an ammonia assay kit; Diagnostic Chemical Limited). Since albumin ($M_w = 68$ kDa) was confined within the ECS (molecular weight cutoff of the HF membrane = 35 kDa), medium samples taken from the ECS at each medium exchange were assayed for human albumin concentration using a commercially available human serum albumin ELISA kit (Bethyl Laboratories).

Calculation of hepatocyte proliferation, metabolism, and varied functions on a per-cell basis

If we assume that cell proliferation in each bioreactor was exponential over the time course of the study (t), the cell growth rate γ for each bioreactor culture can be calculated from the experimentally measured initial and final cell number (N_i and N_f , respectively) using the Monod equation $\gamma = \ln(N_f/N_i)/t$.²⁸ This allows us to calculate the metabolic, synthetic, and detoxification rates on a per-cell basis over the time course of the study.

Quantitative real time (RT)-polymerase chain reaction

RNA was immediately extracted from harvested cells from each HF bioreactor using an RNA isolation kit (SABiosciences, Cat. no. PA-001). RNA (1 mg) was subsequently converted to cDNA using a first-strand kit (SABiosciences, Cat. no. C-03). Duplicate cDNA aliquots from each hepatic HF bioreactor were analyzed on a polymerase chain reaction (PCR) array specific for genes involved in the drug metabolism signaling pathway (SABiosciences, Cat. no. PAHS-002A). The quantitative (q) real time (RT)-PCR analysis was performed on a Cyclar iQ Real-time PCR Detection System (BioRad).

Estimation of maximum O_2 consumption rate and O_2 transport

A mathematical model describing O_2 transport in a HF bioreactor¹⁹ was utilized to estimate the maximum O_2 consumption rate (V_m) of C3A cells within HF bioreactors on the final culture day, with the assumption that the O_2 consumption rate of hepatocytes followed Michaelis–Menten kinetics. The BvHb concentration (15 g/L) in the circulating cell culture medium and the overall cell culture medium flow rate (1.5 mL/min) are fixed parameters in the calculation. Additional experimental data, including the final cell number and HF inlet and outlet pO_2 levels, were also utilized to calculate V_m for each HF bioreactor via a simple trial and error approach, as shown in Figure 2. In this algorithm, an initial guess of $V_m = 0$ was used in the calculation. Other parameters used in the calculations can be found in the literature.¹⁹ O_2 transport in each experimental HF bioreactor can be subsequently simulated given its unique V_m value, which will yield the O_2 concentration profile throughout individual HF's in the bioreactor.

FIG. 2. Algorithm for calculating V_m .

Statistical analysis

All data were presented as the mean $\pm$ standard deviation. Statistical and significant differences between BvHb bioreactors and control bioreactors in the same group (i.e., drug group or no drug group) were determined using student's *t*-test.

Results

Proliferation, metabolism, and varied functions of hepatic HF bioreactors with BvHb supplementation (15 g/L) at 10% O₂

The inlet pO₂ for both control and BvHb bioreactors was 70 ± 9 mm Hg, whereas the outlet was $\sim 33 \pm 7$ mm Hg during the final week of cell culture. The methHb level measured after each medium exchange in BvHb systems was $\sim 25\%$. Figure 3 shows the metabolic, biosynthetic, and detoxification functions of hepatic HF bioreactors on a global and per cell basis over the time course of the study. Overall, the drug group and group with no added drugs almost overlapped in terms of glucose consumption rate, lactate and albumin production rates, and displayed very similar final cell counts.

In Figure 3A and B, the overall metabolic activity of BvHb bioreactors was significantly lower than the controls although both types of bioreactors shared the same growth pattern. In fact, very little glucose consumption and lactate production were observed in both systems until day 7–9, when the metabolic activity started to increase rapidly through the last day of culture. Examining the metabolic activity on a per cell basis (Fig. 3F and G), there is a significant difference between control and BvHb bioreactors.

BvHb systems exhibited a stable metabolic rate (glucose consumption rate $\sim 0.078 \pm 0.021$ mg/h/10⁶ cells and lactate production rate $\sim 0.038 \pm 0.014$ mg/h/10⁶ cells) over the time course of the study, while control systems underwent a remarkable change as the rate of glucose consumption and lactate production nearly tripled within 14 days of culture. The molar ratio of lactate production to glucose consumption in control systems was higher than in BvHb systems in both the drug and no drug groups; however, the difference was not very significant (Fig. 3C).

Figure 3D and H shows no significant difference between control and BvHb bioreactors with respect to albumin synthesis on a global and per cell basis.

In Figure 3E and I, the ammonia removal rate increases sharply with time for both control and BvHb bioreactors after day 11. It was also observed that the ammonia removal rate is significantly higher in the control versus BvHb bioreactors, both on a global and per cell basis.

Figure 3J shows that the amount of hepatocytes recovered from BvHb bioreactors was ~ 55 million (~ 6 -fold expansion), which is significantly higher than the control systems (~ 2 -fold expansion). However, there was no significant difference in the viability of recovered hepatocytes from both systems.

Proliferation, metabolism, and varied functions of hepatic HF bioreactors with BvHb supplementation (15 g/L) at 19% O₂

The inlet pO₂ for all control and BvHb bioreactors was $\sim 127 \pm 6$ mm Hg, whereas the outlet was $\sim 52 \pm 16$ mm Hg

FIG. 3. Metabolic, synthetic, and detoxification functions of hepatic HF bioreactors cultured at 10% O₂. The cell culture medium was supplemented with BvHb at 15 g/L. The solid lines represent the drug group; dashed lines represent the group with no added drugs. (A) Global glucose consumption rate (mg/h). (B) Global lactate production rate (mg/h). (C) Molar ratio of lactate production to glucose consumption. (D) Global albumin synthesis rate (μ g/h). (E) Global ammonia removal rate (μ g/h). (F) Glucose consumption rate per cell (mg/h/million cells). (G) Lactate production rate per cell (mg/h/million cells). (H) Albumin synthesis rate per cell (μ g/h/million cells). (I) Ammonia removal rate per cell (μ g/h/million cells). (J) Final cell count and viability at the end of the cell culture period. All data are shown as the mean $\pm$ standard deviation. $n = 2$ for each group. * $p < 0.05$; # $p < 0.10$ throughout the study if not specified otherwise. BvHb, bovine hemoglobin. Color images available online at www.liebertonline.com/ten.

during the final week of cell culture for the drug group, and $\sim 33 \pm 7$ mm Hg for the group with no drugs. The methHb level measured after each medium change in BvHb systems was $\sim 25\%$. Figure 4 shows the metabolic, biosynthetic, and detoxification functions of hepatic HF bioreactors on a global and per cell basis over the time course of the study. HF bioreactors cultured at 19% O_2 exhibited larger cell mass in BvHb bioreactors than in controls in the same experimental group, which was also observed in bioreactors cultured at 10% O_2 . Higher metabolic activities were observed in the BvHb bioreactor in the no drug group versus the control both on a global and per cell basis (Fig. 4A, B, F, and G, dashed lines). However, similar metabolic rates consisting of glucose consumption and lactate production were found in both control and experimental bioreactors in the drug group. Meanwhile, no significant differences were found for the global albumin synthesis rate and the global ammonia removal rate between control and experimental bioreactors. However, ammonium chloride and drug administration into the bioreactor systems seem to play an important role in determining the hepatocyte proliferation rate. Control bioreactors had a final cell count of ~ 10 million in the drug group compared to ~ 27 million in the group with no drugs, while experimental bioreactors had a final cell count of ~ 23 million in the drug group compared to ~ 56 million in the group with no drugs.

V_m and O_2 transport in each HF bioreactor

V_m was calculated for each group of HF bioreactors on the final day of cell culture (Fig. 5). It was observed that hepatocytes in BvHb-supplemented bioreactors exhibited a larger V_m versus the controls, although the difference in the drug

group was not very significant. Simulated pO_2 profiles throughout an individual HF in each experimental bioreactor system are shown in Figure 6. Each unit represents a cross-sectional view of a single HF including lumen, membrane, and ECS, as shown in Figure 1C. The centerline of the HF bioreactor capillary (lumen) is along the top edge of each figure unit and medium flows from left to right in this representation. It was observed that the concentration of O_2 in control bioreactors was generally higher than that in experimental (BvHb) bioreactors.

An ECS zonation plot was employed to show further details of the distribution of O_2 within the cell-containing ECS (Fig. 7). Oxygenation within the ECS was quantified into the following pO_2 zones¹⁸: hyperoxic (>70 mm Hg), periportal (60–70 mm Hg), pericentral (35–60 mm Hg), perivenous (25–35 mm Hg), and hypoxic (<25 mm Hg) zones. As observed earlier in the pO_2 profile plots (Fig. 6), the ECS of control bioreactors generally exhibited higher O_2 concentrations than those of BvHb bioreactors. At 10% O_2 incubation, *in vivo* O_2 gradients were recapitulated in BvHb bioreactors, with both significant perivenous and pericentral zones and a small periportal zone. On the contrary, control bioreactors displayed a considerable region of the ECS suffering from hyperoxia, and virtually no or very limited perivenous zone. At 19% O_2 incubation, all bioreactors, including controls and BvHb systems, showed significant hyperoxic regions in the ECS due to the high level of the HF inlet pO_2 .

Drug metabolism qRT-PCR

Figure 8A and B showed significant fold changes in expression of genes involved in drug metabolism for bioreactors

FIG. 4. Metabolic, synthetic, and detoxification functions of hepatic HF bioreactors cultured at 19% O_2 . The cell culture medium was supplemented with BvHb at 15 g/L. The solid lines represent the drug group, while dashed lines represent the group with no added drugs. (A) Global glucose consumption rate (mg/h). (B) Global lactate production rate (mg/h). (C) Molar ratio of lactate production to glucose consumption. (D) Global albumin synthesis rate (μ g/h). (E) Global ammonia removal rate (μ g/h). (F) Glucose consumption rate per cell (mg/h/million cells). (G) Lactate production rate per cell (mg/h/million cells). (H) Albumin synthesis rate per cell (μ g/h/million cells). (I) Ammonia removal rate per cell (μ g/h/million cells). (J) Final cell count and viability at the end of the cell culture period. All data are shown as the mean $\pm$ standard deviation. $n=2$ for each group. * $p < 0.05$; # $p < 0.10$ throughout the study if not specified otherwise. Color images available online at www.liebertonline.com/ten.

FIG. 5. V_m in hepatic HF bioreactors maintained at various inlet pO_2 levels (calculated from experimental data based on an existing O_2 transport model). BvHb was supplemented at 15 g/L.

supplemented with BvHb compared to control bioreactors. Most genes involved in phase I enzyme activities were upregulated (2–19-fold changes) in BvHb systems compared to the controls, which consisted of *ADH4/ADH6* (alcohol dehydrogenase), *ALDH1A1* (aldehyde dehydrogenase), and *CYP17A1/CYP1A1/CYP2D* (cytochrome P450 enzyme).

Meanwhile, for biotransformation phase II activity, most of the genes were upregulated (2–125-fold changes) in hepatocytes cultured in BvHb bioreactors, including *EPHX1* (epoxide hydrolase), *GSTA3/GSTZ1/MGST1* (glutathione S-transferase), *NOS3* (endothelial nitric oxide synthase), and *NQO1* (NADPH dehydrogenase). Downregulated phase II genes included *ALOX12* (arachidonate 12-lipoxygenase), *HK2* (hexokinase2), *HSD17B2* (hydroxysteroid dehydrogenase), and *PKM2* (pyruvate kinase).

Transporter genes (group III) and miscellaneous genes (group IV) were downregulated (2–117-fold changes) in BvHb bioreactors as shown in Figure 8A, which consisted of *ABCC1* (ATP-binding cassette), *MT2A* (metallothionein 2A), and *AHR* (aryl hydrocarbon receptor).

Discussion

Metabolism

In cellular metabolism, glucose is consumed and converted to pyruvate via glycolysis in the cytosol. Under aerobic conditions, pyruvate is subsequently oxidized and enters the citric acid cycle in mitochondria to generate 38 moles of ATP from each mole of glucose. In the absence of O_2 , cells only gain 2 moles of ATP from each mole of glucose entering anaerobic glycolysis. Researchers have shown that mammalian cells can trigger a basal metabolic switch for cellular adaptation to hypoxia,²⁹ where aerobic metabolism is blocked and anaerobic glycolysis is shunted toward lactate acid production to compensate for reduced ATP production.

Hepatocytes in our control bioreactors had a much higher glucose consumption rate compared to values in the literature (~0.5 compared to 0.01–0.04 mg/h/million cells^{30,31}) and much higher compared to BvHb systems (~0.08 mg/h/million cells). In addition, higher V_m values in BvHb bioreactors also suggest improved O_2 transport in BvHb bioreactors versus control bioreactors. These results indicated that C3A cells were under hypoxic stress in control bioreactors, which increased glucose uptake resulting from their transition from aerobic to anaerobic metabolism. This assertion was further strengthened by the qRT-PCR results, since genes involved in glycolysis (*HK2* and *PKM2*) were downregulated in BvHb bioreactors. Inadequate O_2 transport in control bioreactors shuts down mitochondrial respiration (where the majority of aerobic metabolism takes place)^{32,33} and promotes glycolysis by increasing glycolytic enzyme gene expression.

Although our computer simulations of O_2 transport in each bioreactor indicate the presence of higher concentrations of O_2 in control bioreactors than in BvHb systems, 15 g/L of BvHb supplementation increased the O_2 carrying capacity of the cell culture medium ~4 times more than the controls. Therefore, on the surface it seems as though the control bioreactors experienced higher levels of oxygenation by only considering the local O_2 concentration; however, these bioreactors are actually suffering from hypoxia, which resulted in a reduced cell mass in the ECS, reduced O_2

FIG. 6. pO_2 profiles in hepatic HF bioreactors maintained at various inlet pO_2 levels (simulated from experimental data based on an O_2 transport model). BvHb supplemented at 15 g/L. Color images available online at www.liebertonline.com/ten.

FIG. 7. ECS zonation in hepatic HF bioreactors maintained at various inlet pO_2 levels (simulated from experimental data based on an O_2 transport model). The vertical axis represents the fraction of space in the ECS that belongs to the various pO_2 zones. (A) Inlet $pO_2 = 80$ mm Hg; (B) inlet $pO_2 = 140$ mm Hg. BvHb supplemented at 15 g/L. ECS, extracapillary space. Color images available online at www.liebertonline.com/ten.

consumption rate, and higher molar ratio of lactate production to glucose consumption compared to BvHb bioreactors.

Biosynthetic function

Albumin plays an important role in maintaining the colloid osmotic pressure of blood and transporting hydrophobic molecules (e.g., drugs and free fatty acids).³⁴ Therefore, it is important to conserve the ability of hepatocytes to synthesize albumin in a HF-based BLAD. The reported albumin synthesis rates for human hepatoma/immortalized hepatocytes (HepG2/C3A/HepLL) ranged from 0.0625 to 0.42 $\mu\text{g}/\text{h}/10^6$ cells^{35–38}; primary human hepatocytes ranged from 0.002 to 0.006 $\mu\text{g}/\text{h}/10^6$ cells³⁹; primary porcine hepatocytes ranged from 0.05 to 6.6 $\mu\text{g}/\text{h}/10^6$ cells^{30,40–42}; and primary rat hepatocytes ranged from 0.2 to 0.4 $\mu\text{g}/\text{h}/10^6$ cells.⁴³ The ca-

capacity for albumin synthesis in our experimental HF bioreactors with C3A cells (0.4–0.8 $\mu\text{g}/\text{h}/10^6$ cells) is comparable or even higher than most of the aforementioned primary cells and cell lines derived from various sources.

Detoxification function

Ammonia is a metabolic byproduct of amino acid deamination and is cytotoxic. In patients with acute liver failure, ammonia can accumulate in the body, which leads to hyperammonemia and hepatic encephalopathy. Ammonia detoxification is thus another essential function a BLAD must recapitulate. A large range of ammonia removal rates have been reported in the literature (0.375–5.57 $\mu\text{g}/\text{h}/10^6$ cells^{38,42,44,45}) for BLAD systems with various sources of hepatocytes. Primary hepatocytes from the pig/rat seem to

FIG. 8. Fold change of genes involved in drug metabolism. The fold change is with respect to BvHb-supplemented bioreactors compared to control bioreactors. Only genes with fold changes >2 above the 90% confidence level were shown in the plots. (A) Group I represents genes involved in phase I activity (oxidation, reduction, and hydrolysis), group III represents genes involved in transporter activity, and group IV represents miscellaneous genes involved in drug metabolism. (B) Group II represents genes involved in phase II activity (methylation, glucuronidation, sulfation, and acetylation). Color images available online at www.liebertonline.com/ten.

have a higher capacity for ammonia detoxification than our systems; however, it is highly possible that the ammonia removal rate in our bioreactors was underestimated at the latter time points of the study as the final ammonium concentration in some of the bioreactors was zero. In addition, the experimental conditions for each study in the literature were also quite different in the measurement of ammonium removal rate in the bioreactor. The initial ammonium concentration (1–5 mM), monitoring time (1–2 days), and total volume of the cell culture medium (100–400 mL) all vary in different studies found in the literature, which makes it difficult to directly compare the detoxification capacity among previous BLAD systems. In the future, more experiments will be conducted at a higher concentration of ammonia in the cell culture medium to better evaluate ammonia detoxification.

Biotransformation—drug metabolism gene expression

BvHb supplementation played an important role in the regulation of drug metabolism, since the expression level of several genes was significantly up- or downregulated.

In the phase I group of enzymes (group I), *ADH4* and *ADH6* encode enzymes in the alcohol dehydrogenase family, which are responsible for catalyzing alcohol metabolism. The *ADH6* gene is also indirectly involved in NADPH production, which plays an important role in the resistance to oxidative stress.⁴⁶ *ALDH1A1* is a member of the aldehyde dehydrogenase family of enzymes, which is abundant in the liver and is the most important enzyme involved in aldehyde oxidation. It was also demonstrated that this enzyme is involved in cellular protection against oxidative stress.^{47,48} In this study, *ADH4*, *ADH6*, and *ALDH1A1* were all upregulated in BvHb bioreactors, indicating a higher capacity for alcohol and aldehyde metabolism in BvHb systems than in control systems. *CYP17A1*, *CYP1A1*, *CYP2D6*, and *CYP2J2* are in the cytochrome P450 family, which are key enzymes involved in drug and toxin metabolism. For an instance, *CYP17A1* is an enzyme involved in steroidogenesis.⁴⁹ The upregulation of cytochrome P450 genes in BvHb bioreactors suggests improved drug metabolism in the cultured hepatocytes.

In the phase II group of enzymes, *GSTA3*, *GSTZ1*, and *MGST1* are from the GST family, which are involved in glutathione homeostasis. All of these genes are upregulated in BvHb bioreactors. *GSTA3* can be induced by the presence of ROS and is thus involved in cellular protection against oxidative stress.⁵⁰ A ~125-fold upregulation of *GSTA3* in BvHb bioreactors indicates a big transition in cellular activity at the transcriptional level. It is possible that increased expression of *GSTA3* is in response to oxidative stress in BvHb systems. *NOS3* is an endothelial nitric oxide synthase, which catalyzes the generation of NO from L-arginine. It has been shown that ROS are involved in the upregulation of *NOS3*.^{51,52} Expression of *NOS3* in BvHb bioreactors was upregulated two to threefold compared to controls, which suggests the presence of higher levels of ROS in BvHb systems versus control systems.

HK2 and *PKM2* (pyruvate kinase M1/M2) both encode glycolytic enzymes and were both downregulated in BvHb bioreactors. *HK2* catalyzes the first step in glycolysis (phosphorylation of glucose into glucose 5-phosphate), while

PKM2 is responsible for the last step in glycolysis where pyruvate is produced. It has been reported that hypoxia promotes glycolysis by upregulating glycolytic enzymes, including *HK2* and pyruvate kinase, via induction of the gene regulator, HIF-1.^{53,54} Downregulation of *HK2* and *PKM2* in BvHb systems complimented the results we observed earlier, where BvHb bioreactors consumed much less glucose than control bioreactors. This result also provides additional evidence of increased anaerobic glycolysis in control systems as a result of hepatocyte exposure to a more hypoxic environment in the ECS versus BvHb bioreactors.

The regulation of *MT2A* (group III) has been considerably studied, but its function is not yet fully understood as it contains several enhancer regions.⁵⁵ There is evidence that hypoxia induces metallothionein expression through metal responsive elements.⁵⁶ *MT2A* was found to be responsive to some heavy metals, including zinc and cadmium.⁵⁷ Considerable downregulation of *MT2A* (up to 117-fold) in BvHb bioreactors strongly suggests that hepatocytes in control bioreactors suffered extensively from hypoxia.

Conclusion

The results of this work demonstrate that hepatocyte proliferation and metabolic function are highly coupled to O₂ availability. BvHb supplementation supports the maintenance of a higher cell mass compared to controls under the same experimental conditions by improving O₂ transport to the ECS. In fact, the maintenance of hepatocytes in BvHb-supplemented bioreactors requires substantially less glucose versus the controls, indicating more efficient cellular metabolism. The capacity for drug metabolism (phase I and phase II biotransformation functions) of BvHb-supplemented HF bioreactors was generally improved compared to controls. Moreover, BvHb bioreactors conserved albumin synthesis and ammonia detoxification (both essential features of a functional BLAD).

Acknowledgments

This work was supported by National Institutes of Health grants R01HL078840 and R01DK070862 to AFP.

Disclosure Statement

No competing financial interests exist.

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Received: February 1, 2010

Accepted: May 24, 2010

Online Publication Date: July 8, 2010