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Normal Atmospheric Oxygen Tension and the Use of Antioxidants Improve Hepatocyte Spheroid Viability and Function

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Abstract

Background—Hepatocyte spheroids have been proposed for drug metabolism studies and in bioartificial liver devices. However, the optimal conditions required to meet the aerobic demands of mitochondria-rich hepatocyte spheroids is not well studied. We hypothesized that an optimal concentration of oxygen could be identified and that the health of hepatocyte spheroids might be further improved by antioxidant therapy.

Methods—Rat hepatocyte spheroids were maintained in suspension culture for 7 days under a mixture of 5% CO₂ plus O₂:N₂ to achieve fractional oxygen contents of 6% (C1), 21% (C2), 58% (C3), 95% (C4). Spheroid health was assessed under each condition by vital staining, TEM, oxygen consumption and mitochondrial counts. Hepatocyte differentiation was assessed by expression of ten liver-related genes (HNF4a, HNF6, Cyp1A1, albumin, Nags, Cps1, Otc, Ass, Asl, Arg1). Functional markers (albumin and urea) were measured. The influence of oxygen tension and antioxidant treatment on the production of reactive oxygen species was assessed by confocal microscopy.

Results—We observed that the hepatocyte spheroids were healthiest under normal atmospheric (C2) conditions with antioxidants ascorbic acid and L-carnitine. Cell death and reduced functionality of hepatocyte spheroids correlated with the formation of reactive oxygen species.

Conclusion—Normal atmospheric conditions provided the optimal oxygen tension for suspension culture of hepatocyte spheroids. The formation and deleterious effects of reactive oxygen species were further reduced by adding antioxidants to the culture medium. These findings have direct application to development of the spheroid reservoir bioartificial liver and the use of hepatocyte spheroids in drug metabolism studies.

Keywords

Spheroid; Oxygen Tension; Hepatocyte Culture; Serum Culture; Serum Free Culture

Introduction

Obtaining well-differentiated hepatocytes with stable functionality is imperative to a biologically active extracorporeal liver support device and to long-term drug metabolism studies. A number of factors have been shown to influence hepatocyte health and functionality of *in vitro* culture systems. For example, our lab and others have shown that primary hepatocytes form spherical multicellular aggregates (“hepatocyte spheroids”) of greater than 40 micron diameter under a variety of conditions including rocked suspension

culture¹⁻⁵. Hepatocyte spheroids possess a 3-dimensional configuration and sustain high rates of viability while maintaining prerequisite function with regards to ureagenesis, albumin production and phase I/II metabolism. As suspension cultures, hepatocyte spheroids offer an ease in sampling and ease in scale-up over attachment culture systems. However, the role of oxygen, the development of deleterious reactive oxygen species (ROS), and the use of antioxidants have not been evaluated in hepatocyte spheroid systems.

The hepatocyte environment *in vivo* is mildly hypoxic owing to its predominantly portal venous blood supply. This observation led some to explore the benefits of a normal oxygen tension environment or a slightly reduced oxygen environment on hepatocyte viability while maintaining critical differentiated function^{6, 7}. Despite this rationale, multiple studies have shown that in most culture conditions low oxygen environments negatively impact hepatocyte viability and metabolism^{8, 9}. This observation may be in part due to the low rates of oxygen provided by diffusion (concentration gradient alone) *in vitro*, a factor that may play a significant role in large hepatocyte aggregates such as spheroids. Furthermore, there is now a significant body of evidence that hepatocytes in their immediate period after isolation consume oxygen at three times their normal *in vivo* rate, though increased oxygen tension has not been shown to benefit hepatocyte function in serum based non-spheroid culture conditions^{10, 11}. Finally, data from our lab has suggested that late death of hepatocyte spheroids may be linked to a caspase independent pathway more suggestive of necrosis than apoptosis¹². This finding suggest that the role of oxygen tension may be different in a well mixed (rocked) spheroid culture system than in other culture conditions leading us to ask the question, does increased oxygen tension in a rocked serum based spheroid culture condition lead to improved hepatocyte health?

Our present study was designed to examine the role of oxygen tension and the use of antioxidants to diminish the deleterious effects of ROS on hepatocyte viability and function in a serum-based rocked culture system. Hepatocyte and spheroid health were examined at oxygen tensions ranging from 40-680 mmHg with and without antioxidants. Mitochondrial counts, gene expression and biochemical activity of the spheroids in each condition were measured over a 7-day period. All studies were performed using rat hepatocytes at a cell density of 5×10^5 cells/ml.

Materials and Methods

Materials

Chemicals were obtained from Sigma–Aldrich (St. Louis, MO) unless stated otherwise. Animals were housed in the Mayo Clinic vivarium and provided ad lib access to water and standard food. All animal procedures were performed under the guidelines set forth by the Mayo Foundation Animal Care and Use Committee in accordance with those set forth by the National Institutes of Health.

Spheroid Cultures and Conditions

Hepatocytes were isolated from male Sprague-Dawley rats (250–300 g; Harlan, Indianapolis, IN) by a two-step perfusion method as previously described¹³ using Collagenase (NB4) (Serva, Heidelberg, Germany). Average harvest viability was $95 \pm 0.8\%$ determined by trypan blue dye exclusion. Freshly isolated hepatocytes were suspended in culture medium composed of William's E supplemented with 10 $\mu\text{g/ml}$ insulin, 5.5 $\mu\text{g/ml}$ transferrin, 5 ng/ml sodium selenite, 10% Fetal bovine serum (Mediatech, Inc., Manassas, VA), 10 U/ml penicillin G, 100 $\mu\text{g/ml}$ streptomycin (Invitrogen, Carlsbad, CA) and 5ng/ml of EGF (SIGMA). Antioxidant supplements (ascorbic acid [4mM], L-carnitine [10mM]) were also added to culture medium as specified. Gas mixtures for spheroid cultures were

obtained using a custom blend (PraxAir) for each study condition. The spheroid culture boxes ($35 \times 30 \times 6$ cm) were custom made of polycarbonate and siliconized with Q7-9170 Silicone Fluid (Dow Corning, Midland, MI). Gas distribution channels were machined in the bottom of the boxes. The bottom of the boxes were covered with a fabric reinforced silicone membrane (silicone thickness of 0.005) to allow uniform gas delivery to the culture box. Gasses were supplied into the channels through polyvinylchloride tubing connected to a supply tank.

Spheroid boxes were placed in the incubator on a modified rocker platform (Bellco Technology, Vineland, NJ) and rocked continuously at a frequency of 10.9 cycles per minute (0.18 Hz) to induce spheroid formation and to maintain spheroids in suspension (Fig. 1). All culture conditions were maintained in a 37°C incubator. In order to achieve equal numbers of spheroids in each test condition freshly isolated hepatocytes from 2-3 rat liver harvests were first pooled in 1 liter of culture medium to achieve an initial cell density of 2×10^6 cells/ml. The pooled suspension of hepatocytes was then rocked in an oxygen tension of 300 mmHg for 24 h to induce spheroid formation. Culture medium was changed after 24 hours when newly formed spheroids were centrifuged, washed and resuspended in 4 liters of fresh culture media. The spheroid suspension was mixed thoroughly to insure uniform distribution of spheroids before equal division into four separate spheroid boxes, each with progressively higher oxygen tension. Each spheroid culture box contained 1 liter of culture medium with or without antioxidant supplements. Final hepatocyte concentration of all boxes was 5×10^5 cells/ml.

Experimental Conditions

In our effort to evaluate the role of oxygen tension on spheroid viability and function we compared spheroids cultivated in medium containing 10% serum at four different oxygen conditions (Table 1): condition 1 (C1) 6% O₂, condition 2 (C2) 21% O₂, condition 3 (C3) 58% O₂, condition 4 (C4) 95% O₂. Oxygen tension (pO₂ mmHg) of each condition was monitored daily after cell inoculation using a GEM Premier 3000 gas analyzer (Model 5700, Instrumentation Laboratory, Lexington, MA). Less than 15% variability in daily measurements of pO₂ was observed over the 6-day studies. To evaluate the role of antioxidants in reducing the impact of ROS a second culture media was created using the same media as above along with the addition of the antioxidants ascorbic acid and L-carnitine (C2+ and C4+). All culture conditions were identical other than differences in oxygen tensions and addition of antioxidants. Each experiment was performed in triplicate.

Immunohistochemistry

Frozen sections of fresh rat liver tissue (4-um serially cut) and whole spheroids obtained after 24 h of formation were immunostained with antibodies against cytokeratin-19 (CK19), hepatic nuclear factor-4 α (HNF4 α), cluster of differentiation-163 or ED2, desmin and the transcription factor GATA4. After antigen retrieval all samples were treated with 3% H₂O₂ and immunostained using proper primary and secondary horseradish peroxidase-labeled antibody (Biocare Medical, Concord CA) (Table 2). Immunohistochemical reactions were developed using diaminobenzidine tetrahydrochloride and 0.01% H₂O₂ and counterstained with Gill's Hematoxylin.

Transmission Electron Microscopy (TEM)

Spheroids were examined by TEM on days 1, 2, 4 and 7 after primary hepatocyte isolation. Briefly, samples of spheroids were preserved in Trump's fixative (1% glutaraldehyde and 4% formaldehyde in 0.1 mol/L phosphate buffer, pH 7.2) and sectioned (90 nm) by the Electron Microscopy Core Facility (Mayo Clinic, Rochester, MN). Sections were placed on

200-nm mesh copper grids and stained with lead citrate. Micrographs were taken with a JEOL 1200 EXII electron microscope operating at 60 kV.

Viability of Rat Hepatocytes

Hepatocyte and spheroid viability and necrosis were evaluated by inverted epifluorescent microscopy (Axioscope, Carl Zeiss Inc., Thornwood, NY) using the Fluoroquench™ fluorescence viability stain (One Lambda, Canoga Park, CA). Green fluorescein isothiocyanate images were captured using a 450-nm excitation filter and merged with in-plane acridine orange images captured using a 546-nm emission filter.

Oxygen Consumption

Three milliliters of culture medium was obtained from each condition and time point with corresponding oxygen tensions immediately measured by a GEM Premier 3000 gas analyzer. Spheroid oxygen consumption was then determined at 2 min intervals over 6 min for each condition on days 1, 2, 4 and 7 using the equation:

$$\text{Oxygen consumption (molO}_2\text{/min)} = (\text{pO}_2\text{.initial mmHg} - \text{pO}_2\text{.final mmHg}) \times (1.29 \times 10^{-9} \text{ molO}_2\text{/ml/mmHg}) \times (\text{volume ml}) / (\text{time min})$$

Mitochondrial Counts

The density of intact mitochondria per cell was determined from representative TEM images of samples collected at baseline and each of the four conditions over the seven-day time course. Image J software (NIH website) was used to estimate the “mitochondria area” and “total cell area” for each micrograph. The ratio of these two areas was then divided by 0.0028 (the ratio of average mitochondrial area to average cell area) to determine the mitochondria count per cell. Average mitochondrial counts were determined from 10 different micrographs and at least five cells (range 5-15 cells) per micrograph at 2500x.

Real-time RT-PCR

Total RNA was extracted from normal rat liver and from rat hepatocytes for each time point and condition using an RNeasy Plus MiniKit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Use of this kit included the removal of residual genomic DNA. The total RNA from rat hepatocytes for each time point and condition was used to prepare and amplify cDNA for the RT-PCR. All PCR were carried out in 10 ul reaction volume using a QuantiFast SYBR Green RT-PCR kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. All amplicons were intron spanning to rule out genomic DNA amplification (Table 3). The specificity of the amplicons was verified by sequencing of RT-PCR products. Data were analyzed with the 7900HT Sequence Detection System (SDS) Version 2.3 software (Applied Biosystems, Foster City, CA) to estimate cycle threshold (Ct) values and enable observation of dissociation curves for characteristic melting curves and temperatures. Expression levels of each target gene were normalized to the expression levels of the endogenous reference gene hypoxanthine guanine phosphoribosyl transferase. The expression levels of each target gene in the day one hepatocytes served as calibrators to determine the relative expression of each target gene at each time point and condition.

Albumin and Urea Measurements

Measurements of urea were performed using a QuantiChrom™ DIUR-500 Urea Assay Kit (BioAssay System, Hayward, CA) per the manufactures instructions. Albumin was measured with a rat albumin ELISA quantification kit from Bethyl Laboratories (Montgomery, TX) per the manufactures instructions. All assays were done in triplicate.

Analysis and Microscopy of Intraspheroid Reactive Oxygen Species

Acetoxymethyl (AM) acetate ester, FM 5-95, and Hoechst trihydrochloride trihydrate staining of spheroids were carried out per manufacture's instructions (Invitrogen, Carlsbad, CA). All staining procedures were followed by immediate examination by confocal microscopy. Spheroids were imaged using an LSM 510 confocal laser scanning microscope (Carl Zeiss Inc., Thornwood, NY) with a 63x water objective (1.2 n.a.) with optical section set to 1.0 μm . Excitation of spheroids occurred using laser wavelengths of 351 nm (Hoechst stain – nuclear), 488 nm (transformed AM molecules – intracellular/intraspheroid ROS), and 546 nm (FM – spheroid plasma membrane). Using the LSM 510 software, spheroids from days 1, 2, 4, and 7 were viewed (C1-C4 were viewed on days 2, 4, and 7). Channel configuration on the LSM 510 software was standardized for the AM stain using day 1 spheroids. This configuration remained constant for all spheroids viewed on days 2, 4, and 7 to allow for consistent comparison between groups. Hoechst and FM- 5-95 stains were used to allow for better visualization of the spatial relationship of the intracellular green AM staining showing the ROS. The channel configurations for the Hoechst and FM stains were adjusted for each condition on each day to achieve optimal images.

Statistical Methods

Results are expressed as mean values $\pm$ SEM. Statistical significance between different culture conditions was determined by paired samples t-test with significance established as $p < 0.05$.

Results

In our effort to evaluate the role of oxygen tension on spheroid viability and function we compared spheroids cultivated in medium containing 10% serum at four different oxygen tensions as summarized in Table 1. Culture conditions were otherwise similar except as noted for antioxidant supplementation.

Cell Composition of Spheroids

To our knowledge no data has been reported previously demonstrating the cell composition of spheroids formed by rocked technique. We examined five primary cell types found within the liver using immunohistochemistry staining for cell specific surface markers— hepatocytes (HNF4a), Kupffer cells (ED2), bile duct endothelial cells (CK19), vascular endothelial cells (GATA4), and stellate cells (desmin). Our results show the presence of hepatocytes, Kupffer cells, and vascular endothelial cells in the isolate immediately after collagenase digestion. However, HNF4a positive hepatocytes were the only cell type seen in formed spheroids after 24 hours (Fig. 2).

Spheroid Health

One method to evaluate hepatocyte and spheroid health is microscopic examination of cellular ultrastructure. TEM of a freshly isolated rat hepatocyte showed abundant mitochondria that were uniform in shape and size before spheroid formation (Fig. 3, Day 0). Healthy hepatocytes and newly formed spheroids (Fig 3, Day 1) revealed intact well-demarcated nuclei with few cytoplasmic lipofuscin bodies and little to no lipolysosomes by TEM. Compromised hepatocyte health and impending cell death were exemplified by ballooning mitochondria, multiple clear lipolysosomes in the cytoplasm and a distorted poorly demarcated cell nucleus of a hepatocyte cultured in hyperoxic conditions (group C4) on Day 7 (Fig 3, Day 7 C4.)

We compared the TEMs and Fluoroquench™ staining of representative spheroids from each condition at day 2, 4 and 7 after hepatocyte isolation and spheroid formation (Fig 4). On day

2, all four conditions showed abundant green-stained (viable) hepatocytes by Fluoroquench™ technique. However, by day 7 hepatocytes maintained in C1, C3 and C4 stained red/orange by Fluoroquench™ indicating that they underwent necrotic cell death. In contrast, most spheroids maintained in C2 conditions continued to show dominant green (viable) staining by Fluoroquench™ on Day 7.

The appearance of representative spheroids in TEM images obtained at corresponding time points support our Fluoroquench™ results. On day 2, TEMs of spheroids obtained from each condition showed good hepatocyte health as demonstrated by their abundant, smooth appearing, non-edematous mitochondria and well-demarcated nuclei. However, TEM images of spheroids from C1, C3 and C4 show significant signs of necrosis and impending cell death on day 7. Mitochondria in these images were markedly edematous or ballooned. In addition, hepatocytes maintained in these hypoxic or hyperoxic conditions showed numerous lipofuscin bodies and lipolysosomes, which were commonly seen in hepatocytes that have died by necrotic cell death. These findings contrasted TEM images obtained from spheroids maintained at physiologic oxygen tension (C2) on day 7, which continued to show smooth mitochondria, few lipolysosomes and distinct nuclei.

Rates of oxygen consumption have been shown to represent overall health of hepatocyte cultures^{14, 15}. Baseline oxygen consumption for C1-C4 was 3.93 $\mu\text{mol}/\text{min}$ on day 1. Oxygen consumption 2, 4 and 7 days after hepatocyte isolation was notably different between spheroids maintained at physiologic oxygen tensions (C2) versus spheroids maintained at hypoxic (C1) and hyperoxic conditions (C4) (Fig. 5a). Spheroids maintained at atmospheric oxygen tension consumed higher amounts of oxygen at each time point compared to the three other conditions. Oxygen consumption was greatest after harvest and declined over time under all conditions.

Average number of intact mitochondria per hepatocyte was 60 ± 10 after spheroid formation. Later values of mitochondrial density are compared to this day 1 value in Figure 5b. C2 showed a significantly higher mitochondrial count compared to the other three conditions at each time point. C2 had an average of 45 ± 18 mitochondria per hepatocyte on day 2, 28 ± 9 mitochondria per hepatocyte on day 4, and an average of 23 ± 6 mitochondria per hepatocyte on day 7. The second highest average mitochondrial count from the remaining three conditions was found in C1. However, mitochondrial counts from all time points in C1 were significantly lower than C2 (day 2, $p = 0.02$; day 4, $p < 0.01$; day 7, $p = 0.03$).

In summary, the rate of oxygen consumption for group C2 has highest at all time points but declined from 60% to 20% of baseline from day 2 to day 7. A decline in mitochondrial count was also observed for group C2 from 45 to 23 mitochondria per cell from day 2 to day 7, respectively. The reason for the greater decline in oxygen consumption than mitochondrial count in C2 is unknown, but may reflect dysfunctional mitochondria which could be counted but were not actively consuming detectable quantities of oxygen.

Hepatocyte Function

To evaluate hepatocyte function from the spheroids maintained at the different oxygen tensions we examined the expression of four liver-specific genes (HNF4a, HNF6, Cyp1A1, Albumin) as well as the expression of six urea cycle genes (Nags, Cps1, Otc, Ass, Asl, Arg1) (Fig 6). Expression levels were measured using qRT-PCR for the four conditions at four time points (Days 1, 2, 4, 7) with day 1 as the reference point. By day 7, C2 had the highest expression levels of all genes investigated (both liver specific genes and urea cycle genes). However, urea cycle gene expression levels decreased steadily under all conditions over the 7-day period. Expression of HNF4a and albumin also decreased throughout the 7-

day time course for each condition. In contrast, the expression of Cyp1A1 and HNF6 remained unchanged or increased slightly with C1, C2 and C3. Of note, the condition with the highest oxygen tension, C4, showed the greatest and/or the sharpest decline in the liver specific transcripts in this study.

Additionally, we examined functionality of hepatocyte spheroids under differing oxygen tensions by the production of albumin and urea (Fig. 7). Urea production by hepatocyte spheroids in C2 was significantly higher than the other 3 conditions ($p<0.01$). Similarly, the rate of albumin production by hepatocyte spheroids in C2 was significantly higher than the other 3 conditions ($p<0.01$). The rate of albumin production remained relatively stable despite a late fall-off in albumin gene expression shown in Figure 6. This discrepancy may represent a continued release of albumin into the culture medium from hepatocytes after loss of their albumin mRNA.

Reactive Oxygen Species in Aging Spheroids

To our knowledge no published data exist linking the development of free oxygen radicals to spheroid health. For each condition (C1-C4) we stained our spheroids at days 1, 2, 4 and 7 with AM, FM 5-95, and Hoechst trihydrochloride trihydrate. All green fluorescence images were captured and processed identically to allow for accurate comparisons. Spheroids sampled 24 hours after primary hepatocyte isolation possessed a scant amount of intracellular reactive oxygen species (ROS) (Fig. 8). Spheroids maintained under atmospheric oxygen tension showed the lowest levels of ROS throughout the 7-day time course. Additionally, both hyperoxic conditions generated the highest levels of intracellular ROS. Day 7 spheroids from the C4 group contained no positive Hoechst blue nuclear staining owing to their absolute non-viable state.

Discussion

The liver, via hepatocytes, is the primary site for lipid, protein, carbohydrate, and drug metabolism in the body. For over two decades a significant amount of effort has been directed at improving culture conditions for primary hepatocytes. Cultured hepatocytes have numerous discovery and therapeutic applications including liver metabolism research and models of infectious disease (viral hepatitis). Primary hepatocytes are also essential to pharmaceutical companies for metabolism and safety studies of new drugs^{16, 17}. Animal models for investigational drug development have failed to accurately reproduce similar effects seen in humans leading to costly and disastrous complications for pharmaceutical companies¹⁸. Some hepatocyte culture systems offer the ability to examine drugs that are rapidly metabolized over a short period of a few hours. However, few systems have been able to provide prolonged hepatocyte health and function to examine slow metabolizing agents and long-term effects of investigational drugs. Additionally, primary hepatocytes also have therapeutic use in extracorporeal bioartificial liver assist devices and in developing cell transplant treatments^{19, 20}. A massive demand exists for hepatocytes with regards to quantity and quality that is not being met with current culture techniques despite tremendous progress towards this end. Therefore, culture conditions for primary hepatocytes will need to be optimized further to better address the above listed needs.

One culture system that has been shown to improve hepatocyte health and function is hepatocyte spheroids produced in rocked, serum-based medium^{4, 5}. However, to the best of our knowledge no report exists to evaluate the role of varying oxygen tensions on hepatocyte health in the spheroid environment. The present study demonstrates that the viability and function of hepatocyte spheroids in a serum-based culture is improved under normal atmospheric oxygen tensions when compared to maintenance in hypoxic or hyperoxic environments. TEM and Fluoroquench™ data showed decreased cell viability and

increased necrosis within spheroids maintained in C1, C3, and C4 when compared to normal atmospheric C2 conditions. Along with the microscopic evidence, we demonstrate that improved hepatocyte spheroid health is associated with significantly higher numbers of intact mitochondria per cell and improved oxygen utilization. As one might expect, spheroid hepatocyte health directly translated into improved overall function. Expression analysis of genes related to hepatocyte function, including urea production, showed a strong trend favoring atmospheric oxygen tensions; the poorest outcomes were found in the highest oxygen tension group (C4). Additionally, absolute urea and albumin production from spheroids in the C2 group were significantly higher than the three other conditions. Finally, a temporal relationship was identified between the development of intracellular reactive oxygen species and poor hepatocyte health.

Others have observed similar results regarding varying oxygen tensions in non-spheroid serum-based hepatocyte cultures ^{6, 9, 10, 15}. In the report by Suleiman et al. it was suggested that components of the serum-based media prevented the expected benefit of using increased oxygen tensions to improve the aerobic environment of the cells due to the formation of deleterious free oxygen radicals ⁶. It is not hard to imagine that the increased formation of free oxygen radicals leads to poor cell health. In this report we show that spheroids maintained under normal atmospheric oxygen tensions exhibit smooth well demarcated nuclei, few areas of frank necrosis and overall significant improvement in viability and function over a seven day culture. Examination of figure 8 shows the expected result that the greatest amount of ROS was produced in the highest oxygen tension environment. In spheroids maintained at the higher oxygen tensions we see loss of nuclei, increasing necrosis, and almost no viable cells by day 7. Since oxygen tension was the primary variable, we conclude that the decreased viability and function of spheroids at higher oxygen tension was a direct consequence of the accumulation of free oxygen radicals; a concept that is well accepted in other culture conditions. In support of this hypothesis we also observed a significant benefit of supplementing the culture medium with two potent antioxidant drugs, ascorbic acid and L-carnitine.

However, a conflict still exists. It is well known that cellular respiration is one of the major rate limiting problems of hepatocyte culture *in vitro*. Hypoxia, though a relative condition, is associated with increased anaerobic metabolism and increased glycogen utilization ^{11, 21, 22}. These changes suggest that providing more oxygen to the culture would improve hepatocyte health. Indeed, beneficial results have been associated with improvements in oxygenation in the setting of the Academic Medical Center bioartificial liver and other extracorporeal liver assist devices ^{8, 23, 24}. However, other reports, including the current study, show that higher oxygen tension environments in serum based cultures continue to confer a negative impact on function and viability ^{6, 9, 25}. A recent report by Kidambi suggests the adverse effects of higher oxygen tension can be obviated in serum free culture conditions ¹⁴. In their study, a significant improvement in hepatocyte function was observed after 13 days in a serum free culture and a hyperoxic environment. These results are encouraging. But then what is the component(s) of serum that contribute to cell death under hyperoxic conditions *in vitro*? Clearly, further research is warranted to address this next question.

In summary, a significant body of evidence exists demonstrating that hepatocytes require sufficient levels of oxygen to maintain their survival and function *in vivo*. Excess delivery of oxygen to cultured hepatocytes comes with apparent risks due in part to the formation of ROS. In our study we have shown that deleterious effects of ROS formation are minimized when hepatocyte spheroids are cultured in normoxic conditions and serum-containing medium supplemented with antioxidants. Serum-free culture conditions may provide further benefit by delivering the much needed oxygen without contributing to the production of high levels of harmful intracellular ROS. Future work must be directed at decreasing the rates of

free oxygen radical formation further and improving their clearance with novel antioxidants and an optimal serum-free medium. Until that day, based on our current results, normoxic conditions minimize the formation of ROS and lead to improved health and vital function of hepatocyte spheroids under well-mixed, rocked conditions.

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Figure 1. 4-Tray Rocked Spheroid Culture System

Spheroids were formed from isolated rat hepatocytes by rocked suspension technique. The left panel demonstrates the rocker platform and 4-tray apparatus inside a cell culture incubator which was used for all experiments. C1 to C4 corresponds to each of the four oxygenation conditions. The upper right panel demonstrates the gas line (GL3) to tray C3. The right lower panel is a light-phase micrograph of five representative hepatocyte spheroids formed by rocker technique.

Figure 2. Immunohistochemistry of spheroid cell types

The five most common cell types native to the rat liver are identified by immunohistochemistry staining: hepatocytes (HNF4a), Kupffer cells (ED2), vascular endothelial cells (GATA4), bile duct endothelial cells (CK19), and stellate cells (desmin) and counterstained with Gill's Hematoxylin. The large background images, captured with a 100x oil objective, show the different cell types from fresh frozen rat liver tissue. The upper left inset images, captured with a 100x oil objective, show the different cell types immediately after collagenase isolation. The lower right inset images, captured with 60x objective, show the different cell types within formed spheroids. Only HNF4a positive primary hepatocytes were identified in spheroids 24 h following collagenase isolation and spheroid formation.

Figure 3. TEM of healthy and necrotic hepatocytes

Day 0 - Healthy hepatocyte immediately after collagenase isolation demonstrating well defined sharp border nucleus (1), organized chromatin, and numerous healthy and uniform mitochondria (small arrow). Day 1 – Healthy hepatocytes within a newly formed spheroid after 24 hours of rocked culture also demonstrating well defined sharp border nucleus (1), organized chromatin, and numerous healthy and uniform mitochondria (small arrow). Necrotic hepatocyte from day 7 spheroid exposed to hyperoxic (C4) environment displaying a distorted irregular nucleus (2) with fragmented chromatin, numerous edematous mitochondria (3), lipofuscin bodies (4), and lipolysosomes (large arrow). Images were captured at 6000x.

Figure 4. TEM and Fluoroquench™ images

Representative images of spheroids from C1 (upper left), C2 (lower left), C3 (upper right), and C4 (lower right) obtained on days 2, 4, and 7. TEM images were captured at 2500x, and epifluorescent images were obtained using an inverted 40x lens. Fluoroquench™ stained hepatocytes were healthy (green) or necrotic (red/orange). Pairing of Fluoroquench™ and TEM images show the progression of cell viability for each condition over the seven-day time course. Spheroids maintained with atmospheric oxygen tensions (C2) displayed the healthiest spheroids at each time point. Measure bars = 10 μ m.

Figure 5. Oxygen consumption rates and mitochondrial counts

Samples were measured on days 2, 4, and 7. The oxygen consumption rates were compared to the day 1 rate of 3.93 $\mu\text{mol}/\text{min}$. Spheroids in C2 had a significantly higher number of mitochondria per hepatocyte at each time point compared to the other three conditions ($p < 0.05$). Spheroids in C2 also showed a trend towards greater oxygen consumption compared to the C1 and C4 conditions at each time point, though this difference was not significant except on day 4 vs. the C4 condition. A benefit of antioxidant therapy was suggested under hyperoxic (C4) conditions during early time points though this benefit did not reach significance.

Figure 6. Expression of Liver Specific Genes

Gene expression of four liver specific genes (HNF4a, HNF6, Cyp1A1, and Albumin) (A) and the six genes involved in the urea cycle (Nags, Cps1, Otc, Ass, Asl, and Arg1) (B) were examined by qRT-PCR during the seven-day time course for each of the four spheroid growth conditions. Day 1 values are the same for all four conditions because all spheroids were formed under the same first 24 h conditions before being divided into C1-C4 groups

Figure 7. Urea and Albumin Production

(A) Average urea production (ug/dL/hr) during days 2 through 7 for each condition was measured using the commercially available QuantiChrom™ DIUR-500 urea assay kit. Measurements of average urea production over the seven-day time course revealed that spheroids in C2 generated significantly more urea when compared to spheroids in the other three conditions ($p < 0.01$). (B) Average albumin production (ug/dL/hr) during days 2 through 7 for spheroids maintained in each condition. The concentration of rat albumin was determined by ELISA which distinguished rat from calf albumin present in the medium. Spheroids maintained under normal atmospheric oxygen tensions produced significantly higher rates of albumin ($p < 0.01$) compared to spheroids maintained under hypoxic or hyperoxic conditions. Production rates of albumin and urea were determined by linear regression fit of measurements made on day 2, 4, 7 of continuous culture. Error bars are the standard deviation of the average of nine calculated rates for each condition.

Figure 8. Reactive Oxygen Species Within Spheroids

Fresh spheroids were obtained from serum-based cultures and sequentially incubated with acetoxymethyl acetate ester (green), Hoechst trihydrochloride trihydrate (blue) and FM 5-95 (red). All images were captured with a confocal microscope using a 63x water objective. The blue Hoechst nuclear stain and the red FM 5-95 plasma membrane stains were used to allow for better visualization of the spatial relationship between the intracellular and extracellular green fluorescent staining of the reactive oxygen species. Confocal settings for the capture of the green fluorescence were identical for each image allowing for accurate comparisons. Green fluorescence represents the presence of reactive oxygen species which was lowest in C2 and greatest in C4 at all time points. As expected, green fluorescence was noticeably reduced with antioxidant treatment of C2 and C4.

Gas Environment of the Four Treatment Groups

Treatment Groups	Gas Mixture and Oxygen Tensions				
	CO ₂ (%)	O ₂ (%)	N ₂ (%)	Theoretical pO ₂ * (mmHg)	Measured pO ₂ ** (mmHg)
C1	5	6	89	43	67±8
C2	5	21	74	142	135±7
C3	5	58	37	414	378±41
C4	5	95	0	677	563±23

* Theoretical measurement based on gas mixture, barometer reading, and H₂O vapor pressure

** Measured in culture medium by blood gas analyzer

Table 1

Table 2

Summary of Immunohistochemistry Conditions

Antibody	Host	Dilution	Incubation	Antigen Retrieval	Supplier
CK19	Goat	1:2000	Overnight, room temp	Citrate buffer at 99°C for 20min	Santa Cruz Biotech, Inc., Santa Cruz, CA
ED2	Mouse	1:100	60min, room temp	None	AbD Serotec, Raleigh, NC
Desmin	Goat	1:100	Overnight, 4°C	None	Santa Cruz Biotech, Inc., Santa Cruz, CA
GATA4	Goat	1:500	Overnight, 4°C	Citrate buffer at 99°C for 6min	Santa Cruz Biotech, Inc., Santa Cruz, CA
HNF4a	Goat	1:250	60min, room temp	Citrate buffer at 99°C for 15min	Santa Cruz Biotech, Inc., Santa Cruz, CA

Table 3

RT-PCR Primer Sequences and Product Length.

Gene	Abbr.	Forward Primers	Reverse Primers	Product Length
Albumin	Alb	GGCACCAAGTGTGTACCCT	AGCACACAGACGGTTCAG	89
Arginase 1	Arg1	CAACACTCCGCTGACAACC	CAGATATGCAGGGGTCAC	126
Arginosuccinate lyase	Asl	TCAACAGTATGGATGCCAACC	CAAATAGGAGATAGCGGTCCT	132
Arginosuccinate Synthetase	Ass	CCAGGAAGAAGGCACTGAAG	GCCTAGGAGATAGCGGTCCT	131
Carbamoyl-phosphate synthetase 1, mitochondrial	Cps1	ACATTGGCTGCAGAATACCC	ACAGCCAGCACCATTATTC	108
Cytochrome P450c (new sequence NM_012540)	Cyp1A1	AGCTAATCAAAGAGCACTACAGG	CCTTATCATCTGAGAGCTGG	128
Cytochrome P450, family 1, subfamily A, polypeptide 2	Cyp1A2	GAGAAGGTGATGCTCTTCGG	ATGCAGGAGGATGGCTAAGA	96
Hepatocyte nuclear factor 4 α	Hnf4a	CCTTGGACCCAGCCTACA	GCTTGAGGCTCCGTAGTGT	175
Hepatocyte nuclear factor 6	Hnf6	CCTGGAGCAAACCTCAAGTCC	CCGTGTTCTTGCTCTTCC	127
Hypoxanthine guanine phosphoribosyl transferase	Hprt	AGGACCTCTCGAAGTGTGG	TCCACTTTCGCTGATGACAC	138
N-acetylglutamate synthase (Human NM_153006)	NAGS	CCGTTGCGGTGCTTCTAGACT	CAGGTTACATTGCTCAGGA	136
Ornithine transcarbamylase	Otc	TGAGGATCCTGCTCAACAAG	ACGGCCTTTCAGCTGTACTT	107