

## Original Article

# Growing a whole porcine liver organ *ex situ* for six hours without red blood cells or hemoglobin

Jing Dong<sup>1\*</sup>, Lingling Xia<sup>1,2\*</sup>, Hefang Shen<sup>1\*</sup>, Congwen Bian<sup>3</sup>, Sujin Bao<sup>4</sup>, Min Zhang<sup>5</sup>, Yiqi Du<sup>6</sup>, Yan Dai<sup>1</sup>, Lijuan Zhao<sup>1</sup>, Yuanhong Xu<sup>5</sup>, Qiru Xiong<sup>3</sup>, Jianjian Xu<sup>7</sup>, Lili Xu<sup>1,8</sup>

<sup>1</sup>School of Basic Medical Science, Anhui Medical University, 81 Meishan Road, Hefei 230032, Anhui, P. R. China; <sup>2</sup>Department of Infectious Diseases, First Affiliated Hospital of Anhui Medical University, Hefei 230032, P. R. China; <sup>3</sup>Department of Chirurgery, First Affiliated Hospital of Anhui Medical University, Hefei 230032, P. R. China; <sup>4</sup>Saint James School of Medicine, Saint Vincent and The Grenadines, <sup>5</sup>Department of Clinical Laboratory, First Affiliated Hospital, Anhui Medical University, Hefei, P. R. China; <sup>6</sup>Department of Anesthesiology, Children's Hospital of Anhui Province, 39 Eastern Wangjiang Road, Hefei 230002, Anhui, P. R. China; <sup>7</sup>Medical Engineering, Hefei University of Technology, Hefei, P. R. China; <sup>8</sup>Department of Cell Biology, SUNY Downstate Medical Center, 760 Parkside Avenue, Brooklyn, New York 11226, USA. \*Equal contributors.

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**Abstract:** Liver transplantation is an effective approach to end-stage liver disease. Shortage of donor liver and increased waiting time for liver transplantation necessitate the development of an organ culture system by which livers can be cultured and maintained *ex situ* for a prolonged period of time. The aim of this work is to test whether cell culture condition *in vitro* could be used to culture whole livers *ex situ* without the use of erythrocytes. Twelve castrated male land race/farm young porcine livers were exposed to 30 min warm ischemia and 30 min cold perfusion. Livers were isolated and connected to an *Ex situ* liver culture system using a standard culture medium RPMI1640 supplied with 10% of fetal bovine serum and sufficient dissolved oxygen under a normothermic condition for 6 hours. Metabolic biomarkers, bile and urea production, hepatic cell viability and histology analysis of biopsies were examined and newly proliferated hepatic cells labeled by BrdU were analyzed after 6 hours *ex situ* culture. The results from biochemical assays and histology analysis indicate that livers after the organ culture still maintain the full function. Conclusions: our data demonstrate that the liver culture system established in this work can be used to culture whole livers *ex situ* in the absence of erythrocytes.

**Keywords:** *Ex situ* liver culture, organ growing, without erythrocytes, 3D culture, BrdU proliferation assay, warm ischemia, BrdU histology analysis, oxygen carrier free

## Introduction

Liver transplantation is a viable treatment option for end-stage liver disease. However, shortages of donor liver and increased waiting time for liver transplantation have caused a rise in mortality in liver disease world-wide. One way to ameliorate this situation is to preserve donor livers for a prolonged period of time before being used for transplantation. Normothermic machine perfusion of the liver holds promise for better preserving and repairing marginal livers. By controlling the culture temperature, oxygen, nutrition, medications, and components necessary for hepatocytes, normothermic machine perfusion provides an *ex*

*situ* organ culture system to maintain liver function. Studies on normothermic machine perfusion, without exception, require either blood [1-4] or hemoglobin [5] as oxygen carriers to mimic hepatic physiological environment *in vivo*.

With red blood cells or hemoglobin, so far normothermic machine perfusion is able to provide the full metabolic support to the liver and create the possibility to evaluate liver viability before transplantation [6, 7].

To date, most cell types have been cultured successfully *in vitro*. Examples include circulating immune cells, stationary tissue cells and

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transformed plasma cells [8]. Currently all successful normothermic machine perfusion procedures for *ex situ* liver organ culture have exclusively used the blood cells as an oxygen carrier [9, 10]. However, tissues do not take oxygen directly from the red blood cells where oxygen is chemically bound to hemoglobin (available from: <http://www.ncbi.nlm.nih.gov/books/NBK54110>). Instead, cells take oxygen from the plasma where oxygen is physically dissolved and released from hemoglobin [11]. In addition, hepatic cells tend to form their *in vivo* original structures under *in vitro* conditions [12]. Moreover, the use of blood as an oxygen carrier also has three other drawbacks. First, the cost for the blood containing medium could be quite high. Second, the blood containing medium has a limited shelf life. Finally, there is a risk for transplant rejection if the blood supplied contains inflammatory cytokines or different major histocompatibility complexes. These issues raise the question of whether the whole liver culture can be maintained without the use of blood cells. Practically, if we adopt the *in vitro* cell culture medium supplied with sufficient oxygen for *ex situ* whole liver culture following a normothermic machine perfusion procedure, will the *ex situ* system be able to maintain the liver under a physiological condition for a prolonged period of time? To address this question, we have attempted to culture porcine livers *ex situ* following a normothermic machine perfusion procedure without the use of blood cells.

### Materials and methods

#### Animals

Twelve castrated male land race/farm young pigs (4-5 kg) were purchased from Guangde County, Anhui, China. All animals were housed and maintained in accordance with Anhui Medical University guidelines for Animals in Research. All experimental procedures and protocols were approved by the "Animal Studies Committee at Anhui Medical University". They were maintained to have access to food and water. The animals were fasted 12 hours with continuous supply of water. Processing of livers from experimental animals began after 30-min warm ischemia and 30-min cold storage followed by 6 hour oxygenated normothermic machine perfusion with cell culture medium.

#### Liver isolation

One hour prior to operation, all animals were injected with 5 mg of diazepam and anesthetized with 3% pentobarbital sodium (40 mg/kg) intramuscularly. The electronic warm blanket was laid under the animals. The skin was clean and the hair on abdominal operation area was removed. A midline abdominal cross incision was performed to gain access to the liver for standard dissection and isolation. The animals were heparinized (1 U/gram body weight). The warm ischemia started from the opening of chest and the cardio was disconnected to the circulation. The gall bladder was removed, and the cannula drainage was positioned 2 cm from common bile duct. The abdominal aorta and inferior vena cava were bluntly isolated. Abdominal aorta closing to the right kidney was blocked, and the cannula was connected into abdominal aorta at the 2 cm below the left renal artery. The supra-hepatic inferior vena cava was cannulated 2 cm above the diaphragm. Thirty minutes later (for warm ischemia), the artery perfusion was started with cold oxygenated RPMI1640 medium (without FBS) and the porta was exposed when stomach was carefully removed, and the portal vein was inserted with a cannula and perfused with the same RPMI1640 medium immediately. The gastric artery and the veins, the splenic artery and the veins, the renal artery and the veins, and the mesenteric arteries and the veins were ligated. The abdominal aorta closed to the diaphragm and its branches were ligated and isolated from the vertebral column. The temperature of liver surface was monitored during the operation (Braun Digital Forehead Thermometer, CVS) and was kept near 4°C. The liver was removed and placed in a bowl (4°C) and connected to *Ex situ* liver culture (ESLC) system (38.5°C).

#### Compositions of the medium in *ex situ* perfusion and ESLC systems

The core medium used *in situ* perfusion was RPMI1640 with controlled temperature and oxygen level. A similar medium was also used for ESLC system with pH adjusted by using 1 M NaOH. Since extracellular matrix components often shelter multiple molecules for cell signaling during inflammation of the liver tissue [13] and only 10% FBS in the medium could influence sheer force to the tissue [14], we increased

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viscosity by adding 1% of low molecule weight dextran [15]. The osmolarity of liver culture medium was monitored by an osmometer (Precision Systems Inc, Natick, MA, USA). Gentamicin (60 mg/L) was added to prevent microbial contamination and atropine (0.1 mg/L) [16] was added to decrease microcirculation resistance in the livers. At 2<sup>nd</sup>, 4<sup>th</sup>, and 6<sup>th</sup> hour of the culture, 20 ml of the medium was used to evaluate liver state. Five liters of the medium without red blood cells were used during ESLC.

### *ESLC system*

ESLC system was particularly designed for liver culture, and it is similar to the liver machine perfusion systems [17, 18], but different in volume and components. ESLC utilized a large volume of medium and we adopted the *in vitro* cell culture medium RPMI1640 as the core medium.

The pressures of the hepatic arteria and the portal vein were controlled by gravity and the resistance of the entrances of arteria and portal vein was measured by Tee water column. The reservoir was placed at the lowest position and the medium was pumped from the lowest reservoir to the highest, and it flowed into each of the intermediate chambers/reservoirs by gravity. The flow order starts from oxygenation reservoir, hepatic artery reservoir, portal vein reservoir, liver chamber, and back-flow reservoir (the lowest reservoir). This flow pattern would supply and maintain each reservoir sufficient medium for liver culture with temperature precisely controlled at 38.5°C.

The oxygenation of medium was made by a device generating very small bubbles (less than 50 µm in diameter) filled with 95% O<sub>2</sub> and 5% CO<sub>2</sub> in the oxygenation reservoir. The content of oxygen in the medium was controlled by a switch connected to a gas cylinder and a device for making bubbles in certain sizes in addition to filters with a cut off of 100 µm in diameter which is sufficient to oxygenation [19]. The oxygenated medium firstly flowed into the hepatic artery reservoir and then through the hepatic artery to the liver by adjustable gravity and slide flow regulator.

### *Monitoring progression of ESLC*

The mean hepatic arterial, the portal vein, and the hepatic vein flow were recorded continuously. Perfusate samples were taken from the

hepatic arterial or the portal vein for flow in and from the hepatic vein for flow out every 2 hours. Samples were analyzed immediately for blood gas parameters (TCO<sub>2</sub>, pCO<sub>2</sub>, HCO<sub>3</sub><sup>-</sup>; pH) and biochemical parameters (Na<sup>+</sup>, K<sup>+</sup>, Cl<sup>-</sup>, glucose, BUN) by I-STAT blood analyzer (Abbott Park, Illinois, U.S.A). In addition, perfusate was collected, frozen and stored at -80°C for evaluation of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) as markers for hepatocytes injury. Albumin was measured as an indicator for hepatic synthetic function. Bile production was collected from a cannula inserted into extrahepatic bile duct and monitored every two hours during normothermic machine perfusion. The bile content (bile salt, bilirubin, LDH) was analyzed. Oxygen levels of the hepatic arterial and the portal vein in flow in and the hepatic vein in flow out were monitored by a dissolved oxygen analyzer (Milwaukee, NC, USA).

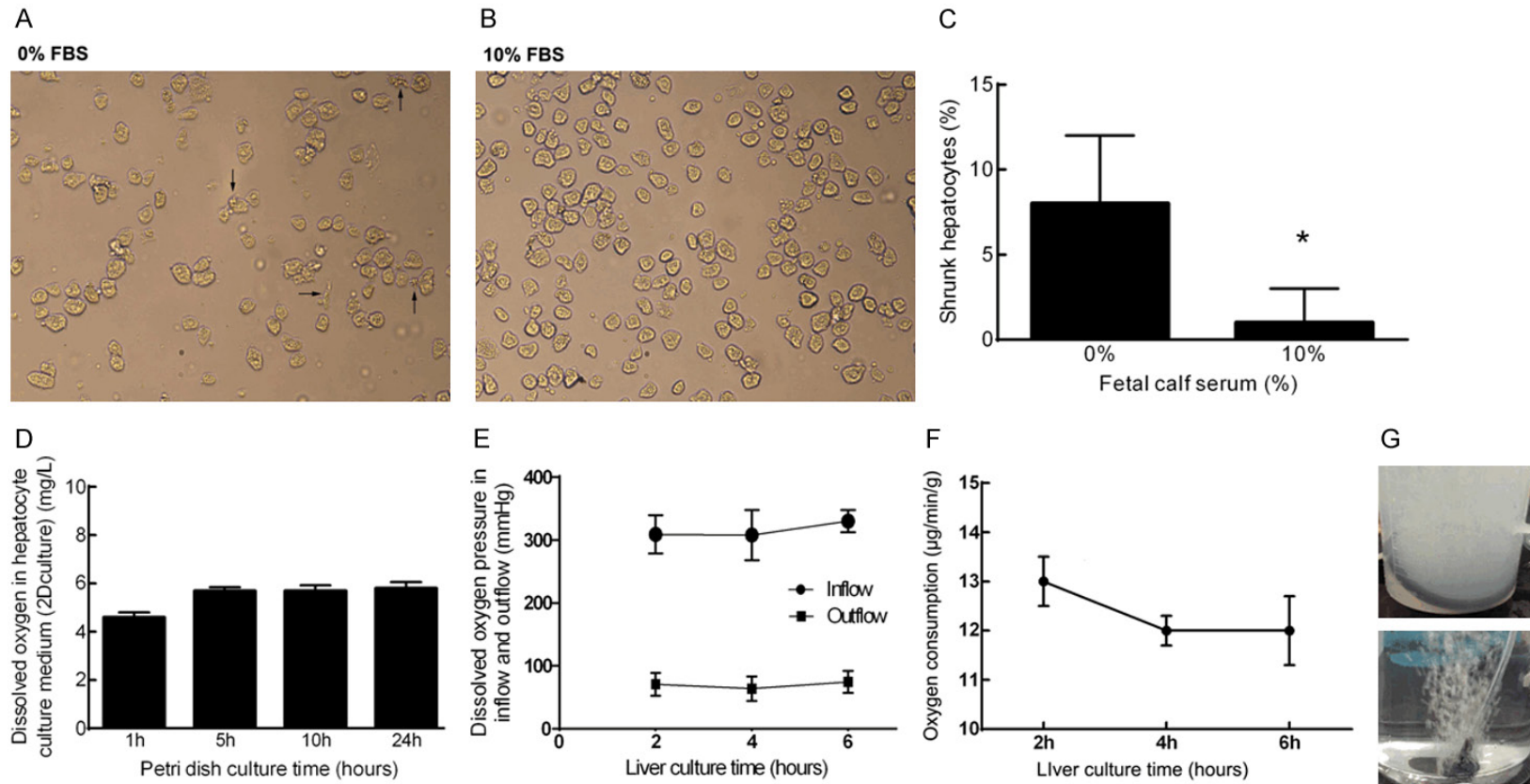
### *Hepatic cell viability in vitro*

After 6 hour perfusion, adequate liver tissues were taken for right lateral lobe. Liver tissues were immediately transferred to a Petri dish with FBS-free RPMI 1640 medium. After gently dispersed by toothed tweezers, the samples were digested by enzymes [20, 21]. The cell dispersion was filtered through a 100 µm pore size cell strainer into a new Petri dish to remove tissue fragments. The single cell suspension was washed 3 times with PBS at 50 g for 5 min at 4°C. The supernatant was aspirated, and the precipitated cells were gently re-suspended in 2 ml RPMI1640 medium (contained 10% FBS). The cells were counted using a hemocytometer and cell viability was determined by trypan blue and the rest of cells with RPMI 1640 medium contained 10% FBS were diluted to 1×10<sup>6</sup> cells/ml and plated at a desired volume on six-well culture plates and cultured at 37°C in a humidified atmosphere supplemented with 5% CO<sub>2</sub>. The cellular viability was examined by trypan blue staining and albumin secretion.

### *ESLC BrdU proliferation assay*

BrdU (5-BRDU) is a thymidine analogue that is incorporated into DNA and routinely and extensively utilized to measure DNA synthesis and to label dividing cells [22, 23]. We took advantage of this to monitor hepatic cell proliferation in ESLC and 1 µM of BrdU (catalog HY-15910, MedChem Express, Monmouth, NJ, USA) was

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**Figure 1.** Hepatic cells cultured *in vitro* and calibration of dissolved oxygen in cell culture and in *Ex situ* liver culture. Cell shrinking was induced by the absence of FBS. A. Representation of hepatic cells cultured in 0% FBS after 24 hours. B. Hepatic cells cultured with 10% of FBS. The black arrows point to the shrunken cells. C. Analyzed results of shrinking cells from the cell cultures. D. The hepatic cell culture medium contains 5.8 mg/L of dissolved oxygen. E. Dissolved oxygen during inflow of the hepatic artery and portal vein; outflow of the hepatic vein was monitored at 2, 4, and 6 hours during ESLC. F. Oxygen consumption of liver tissue is shown during ESLC. G. Representative figure of the devices provide dissolved oxygen at various mmHg values. Cell were accounted 25 fields for each sample with microscope and data are represented as means  $\pm$  SEM (n = 3 independent experiments; \*P<0.05).

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**Table 1.** Biochemical markers in perfusate during ESLC

	ESLC 2 h	ESLC 4 h	ESLC 6 h
Na (mmol/L)	137.50±3.89	139.57±2.44	138.00±2.97
K (mmol/L)	5.32±0.58	5.14±0.38	5.17±0.41
Cl (mmol/L)	110.33±3.72	111.00±4.55	113.67±3.01
Glucose (mg/dL)	146.67±4.25	142.17±6.02	138.20±5.28
TCO <sub>2</sub> (mmol/L)	14.00±0.89	13.29±0.49	12.17±0.75
pCO <sub>2</sub> (mmHg)	28.93±4.18	31.79±3.73	29.23±5.25
HCO <sub>3</sub> <sup>-</sup> (mmol/L)	13.12±0.79	12.29±0.61	11.15±0.82
pH	7.43±0.06	7.36±0.05	7.36±0.07
Lactate acid	Undetectable	Undetectable	Undetectable

used. The antibody for BrdU was purchased from Biolegend (catalog 339802, San Diego, CA, USA). The secondary antibody for mouse IgG1 was bought from Biogenex (catalog QD400-60K, Fremont, CA, USA). BrdU-positive cells were analyzed [23, 24].

### Histological assessment

Biopsies were taken from liver parenchyma before and after ESLC. Biopsies for extrahepatic bile ducts were taken at 6 hour after the start of ESLC. The samples were stored in formalin overnight and then on next day exchanged for 75% ethanol until paraffin embedding. Paraffin-embedded sections were performed at 4 µm of thickness and prepared for hematoxylin and eosin (HE) staining.

### Statistical analysis

Statistical analysis was performed using two-way analyses of variance (ANOVA) and *t*-tests (as documented by columnar graphs) using the GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA). The results are presented as the mean ± standard deviation (SD) and Student's *t* test are used where *p*<0.05 is considered significant.

## Results

### Calibrated dissolved oxygen in porcine hepatic cell culture and in ex situ liver organ culture

To test the feasibility of RPMI1640 in promoting hepatic cell growth *in vitro*, we first examined whether porcine hepatic cells grow in the medium supplied with 10% of FBS (fetal bovine serum). Porcine livers were enzymatically digested [21] and washed three times with RPMI medium (without FBS) and then re-sus-

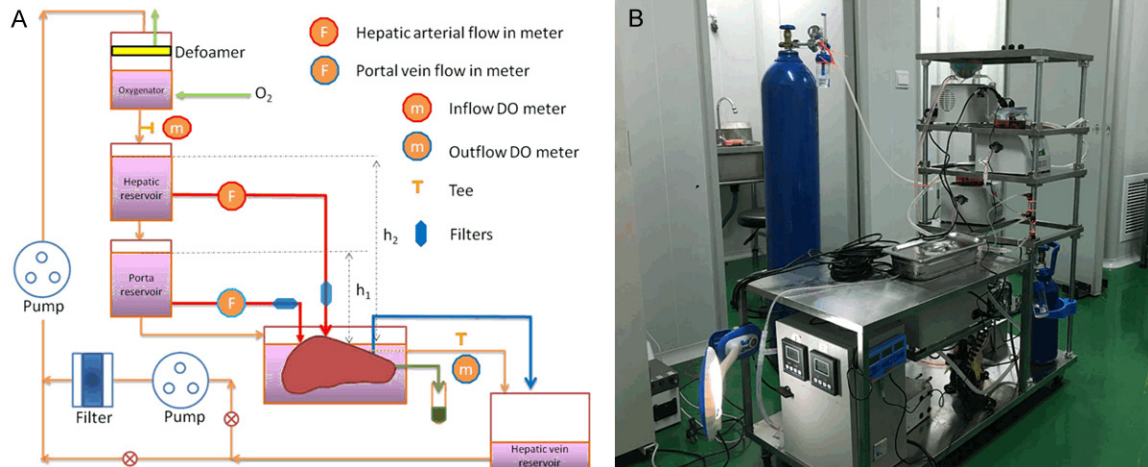
ended in the medium containing 0 or 10% of FBS. Finally the cells were plated at a density of  $0.2 \times 10^6$  per ml in the 24-well plates in triplicate in a standard incubator. In 24 hours, the number of cells in each well was counted with hemocytometer. In the absence of FBS, about 7% of hepatic cells shrank (**Figure 1A** and **1C**), whereas the hepatic cells were healthier in the presence 10% of FBS with less than 1% of shrinking cells detected (**Figure 1B** and **1C**). Simultaneously, we tested dissolved oxygen in the

medium while incubated in the presence of 5% of carbon dioxide. We found that the range of dissolved oxygen was approximately 5.8 mg/L (120 mmHg) at different stages (e.g., 1, 5, 10, and 24 hour after start of the culture) (**Figure 1D**). This result is consistent with those reported from previous studies [25, 26]. Taken together, these data suggest that the oxygen supply at 5.8 mg/L may be sufficient to maintain liver cells in *ex situ* culture.

Given the evidence that  $10^6$  cells required 5 mL of medium containing 5.8 mg/L (120 mmHg) of dissolved oxygen, and the finding that (per gram) liver contains  $1.4 \times 10^8$  cells [27, 28, 29], we estimated that porcine liver would contain  $125 \times 1.4 \times 10^8$  cells and require approximately 88 L of medium (liver weight:  $125 \pm 12$  g) if ESLC system was kept statically. In general, the partial arterial blood oxygen pressure is 100 mmHg (20 ml/dL), in which only 1.5% of the total oxygen in arterial blood is dissolved in plasma and the rest of the oxygen is chemically bound to the hemoglobin of erythrocytes [11, 30] as backup. However, all cells cannot take oxygen directly from hemoglobin in the erythrocytes. Physically dissolved oxygen in the plasma is the only oxygen form that can be taken up by the cells [11]. The actual dissolved oxygen level in the human liver is approximately 30-40 mmHg [31, 32].

We hypothesized that if 40 mmHg of dissolved oxygen in the medium from the hepatic vein outflow could be maintained during ESLC, then liver tissue ischemia should not occur. We performed experiments to create a  $pO_2 > 40$  mmHg in the outflow from the hepatic vein of the liver during ESLC. The oxygen levels of inflow for both the hepatic artery and portal vein were kept at approximately 280-350 mmHg, while

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**Figure 2.** A diagram of ESLC system. ESLC consisted of an organ chamber, two pumps (one centrifugal pump and one roller pump), an oxygenator at the top to maintain oxygen via the hepatic artery/portal vein, and three heat exchangers to maintain the physiological temperature. Two flow meters were installed to monitor the inflow of the hepatic artery and the portal vein. Two online dissolved oxygen meters were also installed for inflow and outflow. Bile production was collected by draining externally (A). The actual ESLC system is shown in (B).



**Figure 3.** Biochemical marker changes in liver organ culture medium. A. Enzymes released; B. Blood urea nitrogen produced during ESLC; C. Albumin production monitored in the medium during ESLC. Data are represented as means  $\pm$  SEM (n = 3 independent experiments).

for the outflow, we found it was about 70 mmHg in the hepatic vein (**Figure 1E**). Oxygen consumption was measured at specific time points during ESLC.

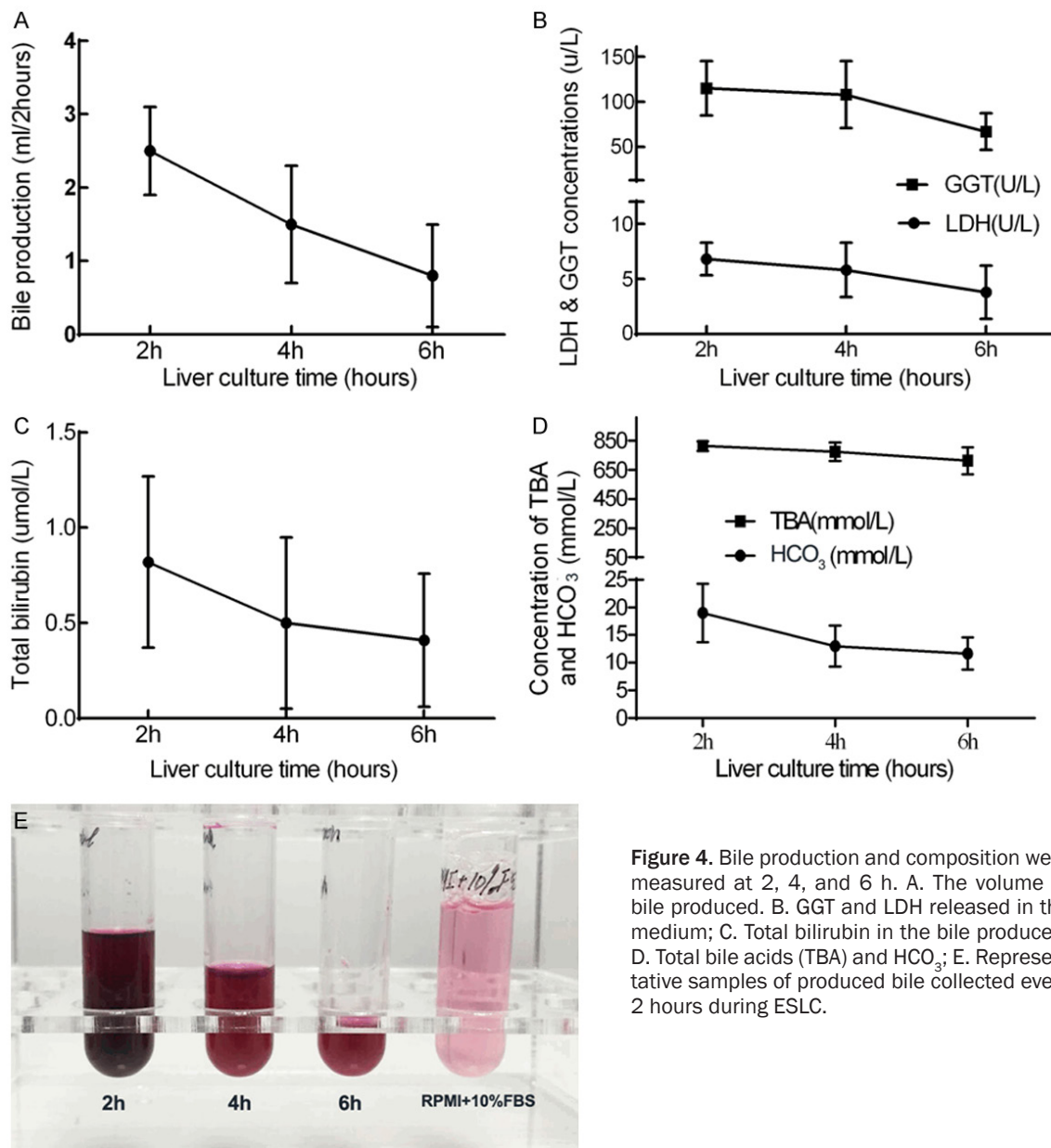
Liver oxygen consumption has been studied in spite of differences in the analytical methods, tissue processing procedures, temperatures by other studies [33, 34]. We evaluated oxygen uptake rate (OUR) calculated as follows:  $OUR = V [CO_2, \text{inflow} - CO_2, \text{outflow}] / \text{liver weight}$  [35], where V is culture medium flow rate (ml/min),  $CO_2$  is the oxygen concentration (mg/l) in the medium. Oxygen consumption was calculated with the concentration of oxygen of the hepatic arteria and the portal vein for inflow as well as the hepatic vein for outflow together with the flow rate. We found that oxygen was consum-

ed at rate of 12  $\mu\text{g}/\text{min}/\text{g}$  by liver during ESLC (**Figure 1F**). The level of lactic acid was kept at  $<0.5 \mu\text{mol}/\text{L}$  during the entire ESLC process, indicating that the liver tissue had taken the oxygen properly (**Table 1**).

### *Ex situ culture characteristics*

The porcine livers were connected to the *in vitro* ESLS by the artery to a pressure tube (50 cm H<sub>2</sub>O) and the portal vein was connected to a pressure tube (10 cm H<sub>2</sub>O) measured by gravity after 30 min for warm ischemia and 30 min for cold perfusion, respectively. Considering that sinusoidal dilatation is easily induced and related to liver injury [36, 37], we reduced the flow from the portal vein so that the hepatic arterial flow and the portal vein flow were main-

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**Figure 4.** Bile production and composition were measured at 2, 4, and 6 h. A. The volume of bile produced. B. GGT and LDH released in the medium; C. Total bilirubin in the bile produced; D. Total bile acids (TBA) and HCO<sub>3</sub>; E. Representative samples of produced bile collected every 2 hours during ESLC.

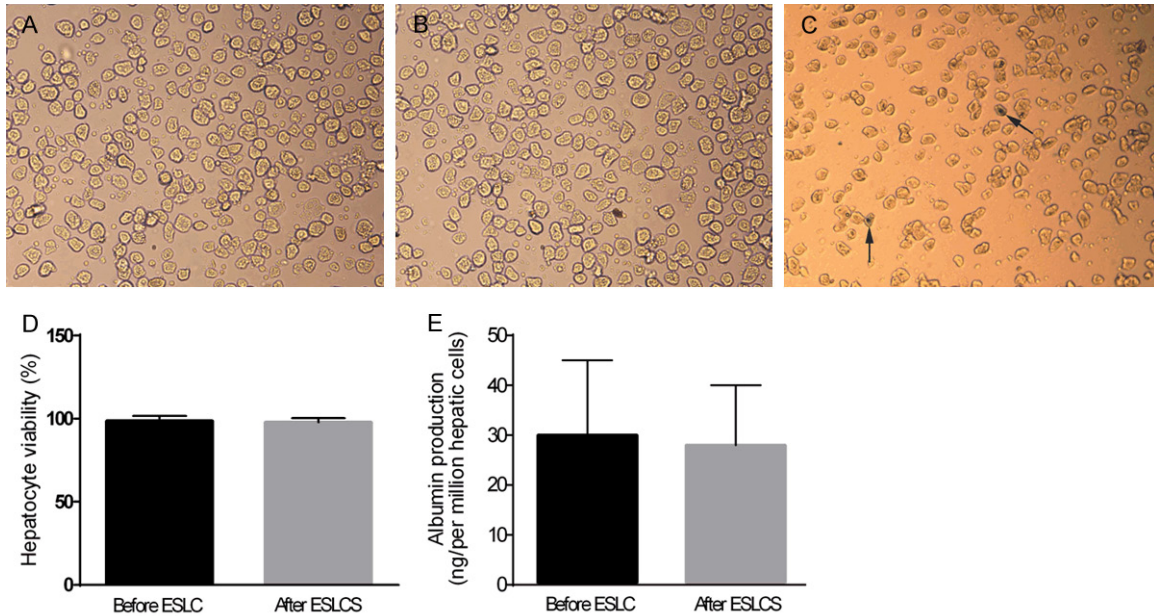
tained at 45% (40-50 mL/min) and 55% (50-60 mL/min) of the total flow, respectively. The pressures for the portal vein and hepatic artery were stably maintained at 10 and 50 cm H<sub>2</sub>O, respectively. A diagram of the ESLC system and the actual system are shown (Figure 2).

### Metabolic biomarkers

The mean hepatic artery, portal vein, and hepatic vein flow rates were continuously recorded. The culture medium (perfusate samples) were taken from the hepatic artery or the portal vein for inflow and from the hepatic vein

for outflow every 2 hours, and they were immediately analyzed for blood gas parameters (TCO<sub>2</sub>, pCO<sub>2</sub>, HCO<sub>3</sub><sup>-</sup>, and pH) and biochemical parameters (Na<sup>+</sup>, K<sup>+</sup>, Cl<sup>-</sup>, glucose, and BUN) (Table 1). Some samples were also collected, frozen, and stored at -80°C until evaluation for ALT and AST, which served as markers of hepatic injury (Figure 3A). Albumin was measured as a marker of hepatic protein synthetic function (Figure 3C). Given that urea is synthesized in the liver and the kidney of many vertebrates as part of the urea cycle [38], urea is only synthesized by the liver in our ESLC and can be used as an indicator of liver function [39-42]. We

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**Figure 5.** Hepatocyte viability and albumin production from hepatocytes isolated before and after ESLC. A piece of the liver tissue enzymatically digested and Trypan blue stained before ESLC (A) and after ESLC (B). (C) Trypan blue positive for cell control was compared in the testing experiment. (D) Viability of hepatic cells as assayed using trypan blue before and after ESLC. (E) Albumin production per million cells isolated before and after ESLC cultured in Petri dishes. These data are shown as the mean  $\pm$  STDEV ( $n = 5$ ).

monitored urea production throughout the entire culture (**Figure 3B**) and found that the urea concentration in the medium steadily increased during ESLC. Remarkably, in the final hours, the urea concentration was above the upper limit of the normal range (1.2-3.0 mmol/L) [43] (<http://www.ncbi.nlm.nih.gov/books/NBK305>). For conversion,  $\text{BUN (mg/dL)} = \text{BUN (mmol/L)} \div 0.375$ ;  $\text{Urea (mg/dL)} = \text{BUN (mg/dL)} \times 2.14$ . BUN was 10 mg/dL in the culture medium after 6 hours of ESLC, which was very close to the human physiological range.

### Bile was produced during ESLC

Bile production is another indicator for the liver function. The major components of the bile are bile acids derived from cholesterol [44]. We monitored the bile production and total salts contained in the bile in every two hours during the entire ESLC. The mean rate of bile production was  $4.5 \pm 0.9$  ml/per liver during the entire 6 hours of organ culture. The amount of bile produced was  $2.5 \pm 0.6$  ml and  $1.2 \pm 0.5$  ml at 2 and 4 hour, respectively, after the start of ESLC. The last two hours was only  $0.8 \pm 0.4$  ml (**Figure 4**). The efficiency of bile production was 60% of human physiological bile product at the first two hours [45]. The efficiency of bile production

decreased to 23% and 15% at 4 and 6 hour, respectively, after the start of ESLC. The bile content (bile salt, bilirubin, LDH) was analyzed and the bile level declined slightly during 6 h of ESLC (**Figure 4**).

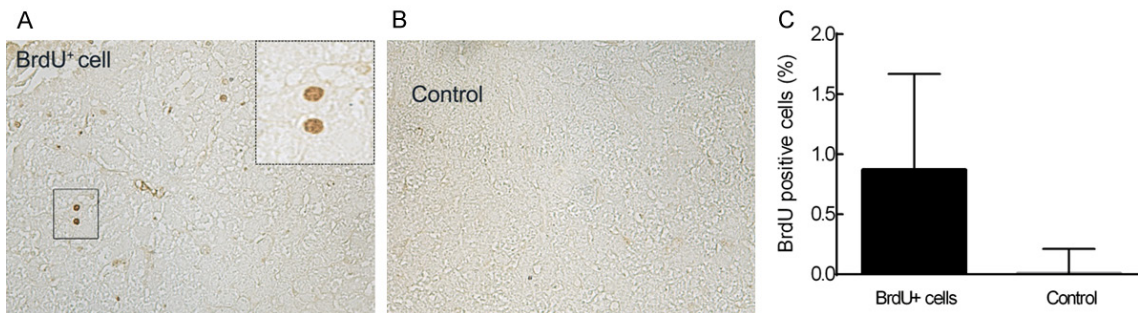
Bile produced by livers *in situ* appears to be greenish in normal individuals. We notice that the bile we collected during ESLCS was green at the beginning and gradually became reddish. RPMI medium contains the phenol red as an indicator for pH. In addition, it is known that phenol red is cleared up by livers in a rat model [46]. Therefore, it is likely that the phenol red was taken up by the livers during ESLC.

### Hepatic cell viability

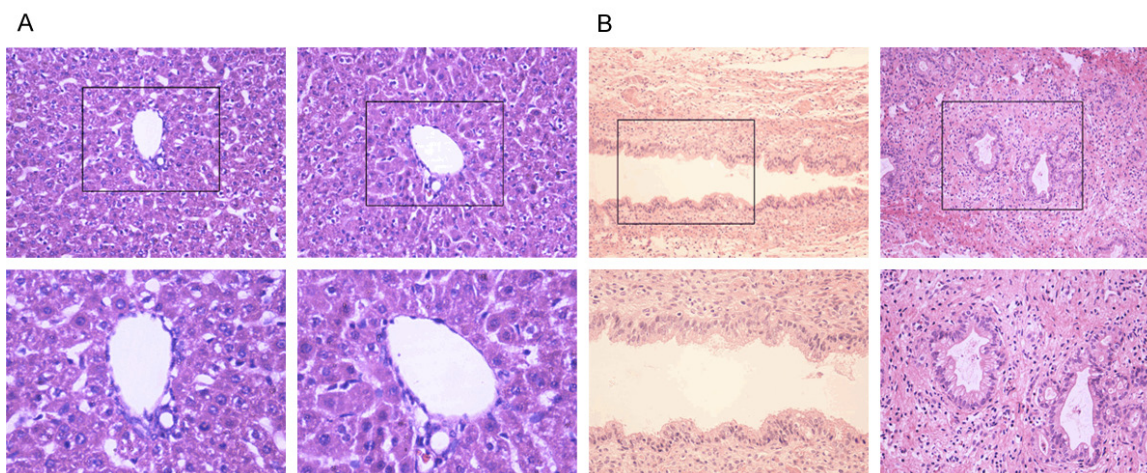
Hepatic cells isolated from livers before and after ESLC were evaluated for cellular viability by trypan blue staining and albumin production. Isolated hepatic cells were subject to *in vitro* culture for 24 hours and cellular viability was performed subsequently. The viabilities were 99.6% and 98.6% for hepatic cells isolated before and after ESLC, respectively (**Figure 5**), indicating no significant differences in the viability between samples collected before and after ESLC. The albumin production in the su-



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**Figure 6.** BrdU hepatocyte proliferative assay in ESLC. The representative tissue containing BrdU-positive cells taken after 6 hours of ESLC is shown in (A), and two newly proliferated neighboring positive cells (black box with broken lines) was enlarged and inserted on the right side. (B) showed the experimental negative control and (C) is the analyzed number of BrdU-positive hepatocytes in comparison with control. These data are shown as the mean  $\pm$  STDEV (n = 3).



**Figure 7.** HE staining of the liver and bile duct biopsies following 6 hours of ESLC. A. HE staining of the liver tissues before ESLC (left column) and 6 hours after ESLC (right column). The top panel shows the hepatic architecture of the liver tissue (magnification  $\times 200$ ), while the bottom row shows enlargement of the center vein with a completely enclosed endothelial layer. B. Histology of the bile duct and peribiliary glands after 6 hours of ESLC. The top row shows the morphology of the common bile duct (upper left) and peribiliary glands (upper right). The bottom row shows an enlarged common bile duct (lower left) and enlarged peribiliary glands (lower right).

pernatant of cultured hepatic cells isolated from livers before and after ESLC was also quite similar (**Figure 5E**). Specifically, per million of cells, 30 ng albumin was produced by hepatic cells isolated before ESLC and 28 ng produced by an equivalent number of those isolated after ESLC (**Figure 5E**). These results support that viability of hepatic cells after ESLC has not been compromised.

### *Hepatocytes proliferated during ESLC*

BrdU cell proliferative assay can directly indicate which cells are newly proliferated during ESLC. Biopsies of each cultured liver were sliced and processed. BrdU incorporated in new-

ly synthesized proliferative cells was recognized by the antibodies (**Figure 6**). The number of BrdU-positive cells was approximately 1.5%, whereas control (missing antibody for BrdU or the secondary antibody) had about zero positive cells. These results indicate that the ESLC system, which did not feature red cells or hemoglobin, successfully provided a potentially suitable environment for liver regeneration.

### *Liver and bile duct histology*

Finally, to examine hepatic cells at a cellular level, we performed liver biopsy. Samples of livers after 6 h of ESLC were processed and stained with hematoxylin and eosin (HE). Liver

biopsy samples taken before ESLC were used as a control. We did not find any significant morphological differences in sinusoidal spaces from the biopsies taken before and after ESLCs (**Figure 7A**). As integrity of bile duct and peribiliary glands are important for liver regeneration [47, 48], we also examined histological morphology of biopsies from extrahepatic bile duct. We found that the entire biliary epithelial layer was properly laid in the common bile duct as well as the peribiliary glands (**Figure 7B**). There were no significant histological differences in the bile duct and peribiliary gland in liver biopsies taken before and after ESLC (**Figure 7B**). Therefore, these results support that no significant injury was done to the livers during ESLC.

### Discussion

Our data demonstrate that cell culture medium oxygenized with suitable flow rate is capable of maintaining the liver function for at least 6 hours. We believe that the functional livers had been supported by optimal microcirculatory environment, since all hepatic cells such as hepatocytes, hepatic endothelial cells, kufferr cells, and stellate cells were carefully kept at their original homes. To our knowledge, this is the first study in liver research showing that liver function can be maintained in *ex situ* organ culture without the use of erythrocytes.

Bile production is an indicator for liver function. During ESLC, we noticed that bile production peaked at  $1.3 \pm 0.3$  ml/100 g/hour in the first two hours which equals 60% of physiological production in humans if 800 ml of bile is produced by 1500 grams of human liver daily [45]. In the cultured liver, bile production decreased to  $0.5 \pm 0.2$  ml/100 g/hour during the second two hours and to  $0.25 \pm 0.2$  ml/100 g/hour during the last two hours in all livers we tested so far. Interestingly, the color of the bile was darker at the beginning and gradually becoming lighter and lighter. At fourth hour during ESLC the bile became reddish and dark reddish. These color alterations in bile production might be due to phenol red in the RPMI medium. Without hemoglobin in the system the reddish color in the bile very likely came from phenol in RPMI, since phenol is secreted in biliary production by the liver [46, 49]. These color changes indicate that phenol in RPMI could be used as a parameter to evaluate liver function in our ESLC system, though phenol may cause harm-

ful effects on the central nervous system in vivo.

Another important parameter of liver function is urea production. In ESLC system there was no way to remove urea unless the medium was changed, since the kidney was not present in the system. Therefore, the steady rise of urea production in ESLC serves as an indicator that the liver was kept in a proper state. At the beginning urea was undetectable ( $<3$  mg/dL). During the second two hours, BUN 6 was detected (to convert BUN into urea multiply by 2.14). BUN peaked at 10 mg/dL by the end of one experiment. Considering the volume of the medium used in ESLC, we calculated that urea was produced at 2140 mg/100 g/h, which were the highest in the literature reported to date. Those data support that the livers during ESLC were functional.

ESLC system using cellular culture medium has several advantages over erythrocyte-based culture systems. First, cellular culture medium is inexpensive compared with blood-based media. Second, the cellular culture medium has a low risk of infectious pathogens. As a result, the cellular medium has much longer shelf life than erythrocyte-based media. Finally, there were no foreign immunological or cellular factors such as antibodies or inflammatory cytokines (e.g, IL-1 and IL-6) involved. Without those factors, use of cellular culture medium in ESLC further lowers the risk of reperfusion induced injury.

Despite the significant advantages of ESLC we have described herein, we should point out that several issues still remain to be addressed. First, any side-effect of oxygen at the levels we used has not been tested. Clearly, our results from liver functional evaluations after ESLC indicate that oxygen was properly supplied when dissolved oxygen at 70 mmHg from out-flow of hepatic vein. Whether the levels of oxygen we used have caused any oxygen toxicity to the liver during organ culture currently is not clear. Second, we used RPMI medium supplied with 10% of FBS in this work. However, we still don't know whether this medium is optimal for whole liver culture. Third, our results support that the livers were functional in our biochemical assays. We have not tested whether livers subject to ESLC for 6 hours are still in a native state so that they can be used immediate-

ly for liver transplant. These issues should be addressed in future studies.

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## Disclosure of conflict of interest

None.

## Abbreviations

ESLC, *Ex situ* liver culture; FBS, fetal bovine serum; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; HE, hematoxylin and eosin; SD, standard deviation; ANOVA, analyses of variance; RPMI, Roswell Park Memorial Institute.

**Address correspondence to:** Dr. Jianjian Xu, Medical Engineering, Hefei University of Technology, P. R. China. Tel: 13309699846; E-mail: hfgdxjj@126.com; Dr. Lili Xu, School of Basic Medical Science, Anhui Medical University, 81 Meishan Road, Hefei 230032, Anhui, P. R. China; Department of Cell Biology, SUNY Downstate Medical Center, 760 Parkside Avenue, Brooklyn, New York 11226, USA. Tel: 1-914-316-0801; E-mail: lili\_xu@optonline.net

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