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Original Article

Biliary epithelial cells proliferate during oxygenated *ex situ* liver culture

Congwen Bian[1*], Yiqi Du[2*], Rui Ding[3], Jun Huang[3], Yan Dai[4], Sujin Bao[5], Lijuan Zhao[4], Hefang Shen[4], Jing Dong[4], Jianjian Xu[6], Qiru Xiong[1], Lili Xu[4,7]

[1]Department of Chirurgery, First Affiliated Hospital, Anhui Medical University, 218 Jixi Road, Hefei 230022, Anhui, P. R. China; [2]Department of Anesthesiology, Children's Hospital of Anhui Province, 39 Eastern Wangjiang Road, Hefei 230002, Anhui, P. R. China; [3]Department of Chirurgery, Third Affiliated Hospital, Anhui Medical University, 390 Huaihe Road, Hefei 230009, Anhui, P. R. China; [4]School of Basic Medical Science, Anhui Medical University, 81 Meishan Road, Hefei 230032, Anhui, P. R. China; [5]Saint James School of Medicine, Saint Vincent and The Grenadines; [6]Medical Engineering, Hefei University of Technology, 139 Tunxi Road, Hefei 230009, Anhui, P. R. China; [7]Department of Cell Biology, SUNY Downstate Medical Center, 760 Parkside Avenue, Brooklyn, New York 11226, USA. [*]Equal contributors.

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Abstract: Biliary complications remain a major source of morbidity in liver transplant patients. Among these complications, nonanastomotic biliary strictures (NAS) are especially common and they are frequently therapy resistant in part because biliary epithelial cells are more sensitive to warm ischemic injury than hepatocytes. It has been a challenge to maintain the physiological function of biliary epithelial cells during liver transplantation. In this work, we have examined the effect of oxygen on proliferation of biliary epithelial cells in the rat livers obtained from donation after circulatory death (DCD). Twelve rat livers from DCD were divided into two groups. Livers in the control group were isolated following a standard procedure without oxygen supply. Livers in the experimental group were isolated with a constant supply of oxygen. All livers were then connected to an *ex situ* liver culture system in the presence of bromodeoxyuridine (BrdU), a thymidine analogue and a marker for cell proliferation. After 6 hours of normothermic *ex situ* liver culture, morphology and DNA replication in hepatocytes and biliary epithelial cells were assessed and compared between the two groups. We found that about 4.5% of the biliary epithelial cells in the experimental group proliferated compared with only 0.4% of cells in the control based on BrdU staining. No significant change in cell morphology was observed in those cells between the two groups. Thus, our results indicate that oxygen supply is required for maintenance of the physiological function of biliary epithelial cells during liver transplant and suggest that a constant oxygen supply during liver isolation along with *ex situ* liver organ culture can enhance the repair of biliary epithelial cell injury during liver transplantation.

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

Introduction

Despite the fact that graft survival rates of livers from donation after brain death are higher than from donation after circulatory death (DCD), livers obtained from DCD have been increasingly used for liver transplantation due to shortage of donor livers [1-3]. It is known that DCD livers have a higher risk of biliary complications [4-6]. Biliary complications are a major cause of morbidity after orthotopic liver transplantation (OLT). Among these complications, nonanastomotic biliary strictures (NAS) are especially common and occur in as many as 30% of liver recipients after OLT. Up to 50% of the patients with NAS require retransplantation, since NAS are frequently therapy resistant [4].

Biliary epithelial cells are more vulnerable to warm ischemic injury than hepatocytes [7], which may account for a high incidence of NAS even after a successful DCD liver transplantation [8]. Anatomically, hepatocytes are supported by double blood supplies, i.e., the portal vein and hepatic artery. The intraepithelial and ex-

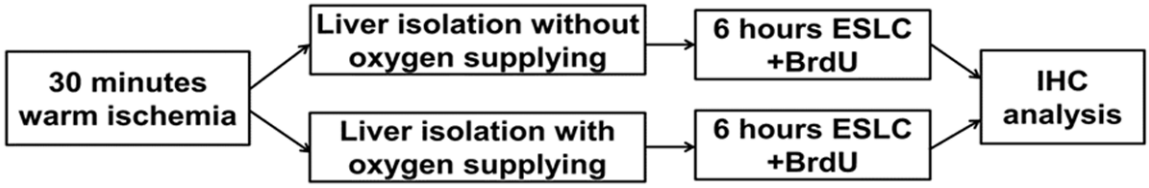

Figure 1. Schematic representation of the experimental design adopted to assess the effects of oxygen supply during liver isolation on the liver function. Six rat livers were used for each group.

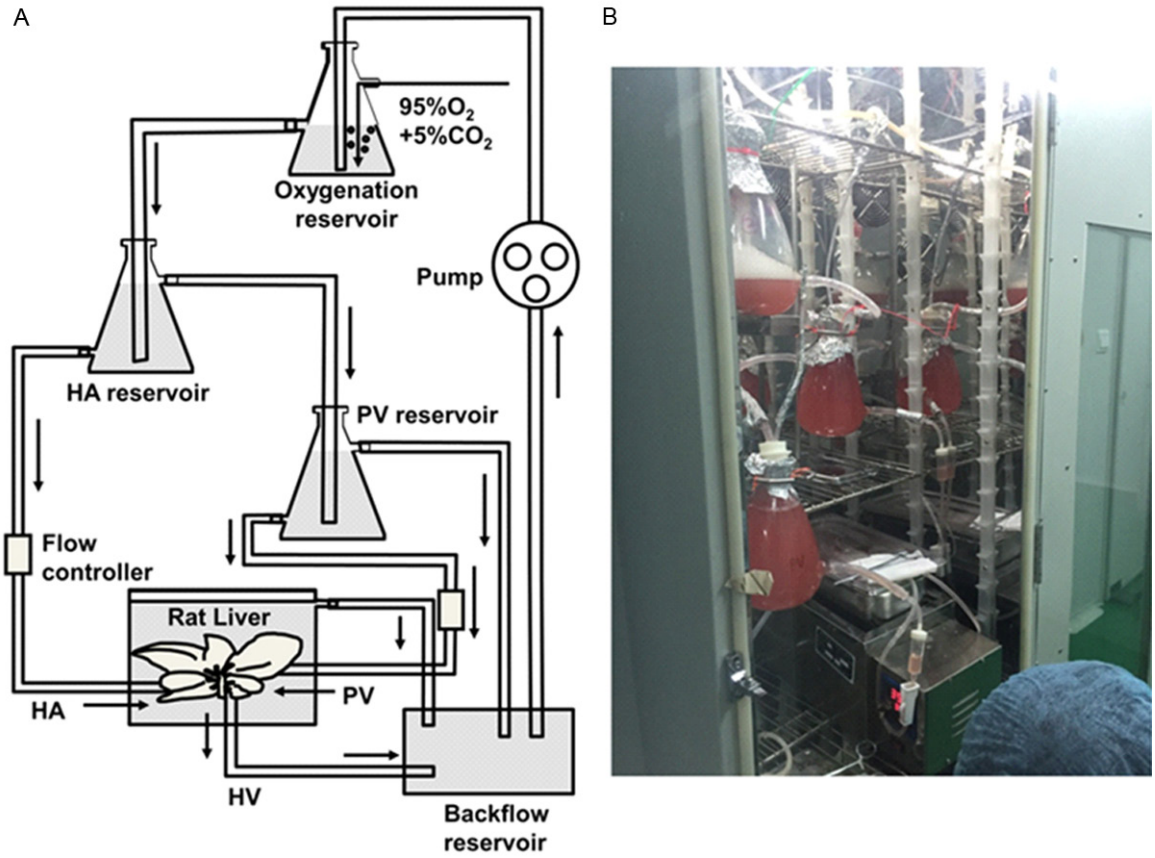

Figure 2. The ESLC system for rat livers. A. A diagram of the ESLC system. It consisted of an organ chamber, one pump (one roller pump), an oxygenator at the top to maintain oxygen via the hepatic artery/portal vein. The entire system was kept at the physiological temperature in an incubator. B. The actual ESLC system.

traepithelial cells within the biliary epithelium mostly depend on the hepatic artery. In addition, second warm ischemia or warm ischemia in recipient often occurs due to a rise in the temperature of the liver when hepatic artery anastomosis is performed after the portal vein is unclamped during implantation [6, 7].

Normothermic machine perfusion holds promise for repairing liver injury *ex vivo*, since it keeps the liver graft at a physiological temperature and supplies adequate oxygen to maintain aerobic metabolism [9]. Our previous work has shown that the hepatocytes proliferate during the culture *ex vivo* [10]. This study is focused on the effect of oxygen on the maintenance of biliary epithelial cells of liver graft, and our data suggest that a sufficient oxygen supply may provide a more optimal environment for the biliary epithelial cells to regenerate.

Material and methods

Study design

To study whether livers consume oxygen at a low temperature, we set up a system to measure oxygen consumption in the liver. Twenty

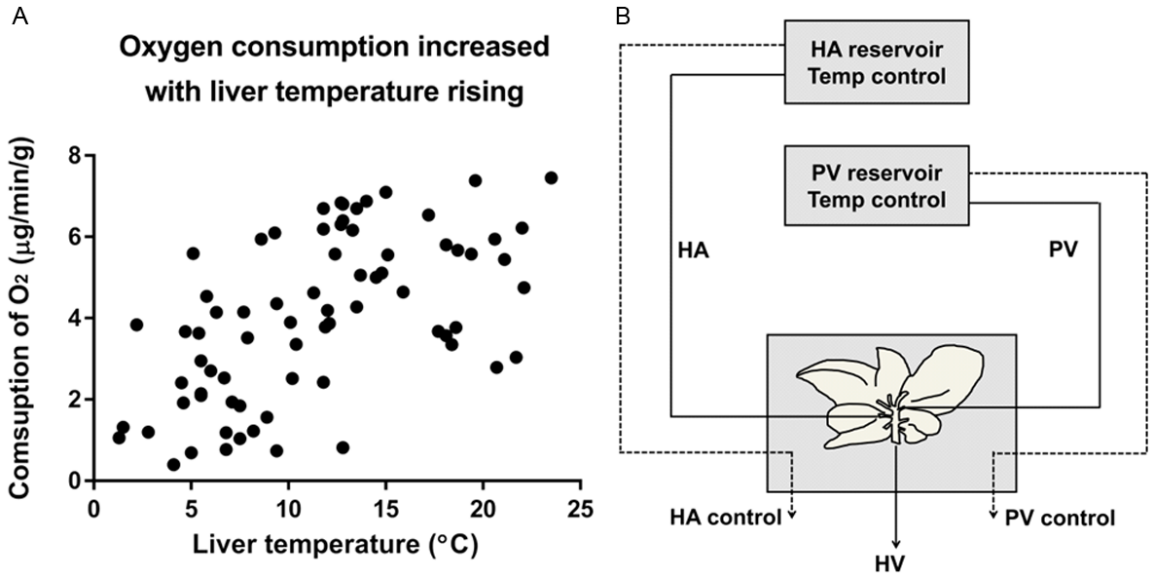

Figure 3. Livers consumed oxygen at different temperatures. A. Livers tended to consume more oxygen at higher temperatures. B. A diagram of the system for measuring liver oxygen consumption. It consisted of an organ chamber and two reservoirs (one for hepatic arterial perfusion and one for portal vein perfusion). The broken lines show the parallel controls for both the hepatic artery (HA) and the portal vein (PV).

rats were used in this measurement to quantify oxygen consumption of the liver at different temperatures. These livers were discarded after the measurement. To investigate the effect of oxygen on the hepatocytes and biliary epithelial cells, other 12 male rats were used and they were divided into two groups: the oxygen supplying group (the experimental group) included livers that were isolated with a constant supply of oxygen following 30 minutes of warm ischemia; accordingly, livers that were isolated without oxygen supplying were used as control. All livers in the two groups underwent *ex situ* liver culture (ESLC) for six hours using RPMI 1640 with 10% of fatal calf serum in the presence of BrdU [10]. Schematic representation of this experiment to test the impact of oxygen on hepatocytes and biliary epithelial cells is shown in **Figure 1**.

Liver isolation

A total of thirty two male Sprague Dawley rats (280-300 g) were used in this work. Animals were maintained in the Anhui Medical University. Animals used in all these experiments were approved by the Animal Ethics Committee at Anhui Medical University. Anesthesia was performed using pentobarbital sodium (50 mg/kg i.v.) and isoflurance with an anesthesia machine. An incision on midline abdominal was performed to gain access to the liver following a standard dissection and isolation procedure [10, 11]. Heparin was used at approximately one unit per gram body weight. Thirty minutes of warm ischemia following the opening of chest, the gall bladder was removed, and the inferior vena cava and the abdominal aorta were bluntly isolated. The artery and the portal perfusion was conducted with cold RPMI 1640 medium either oxygenated or non-oxygenated [10, 11]. The periphery arteries and the veins of the liver were ligated. The liver was stored in a bowl at 4°C. For *ex situ* liver culture, each liver was connected to the ESLC system that was maintained at 38.5°C.

Oxygen consumption of the liver at different temperatures

Measurement of oxygen consumption was performed following a procedure described previously [10, 12]. The system used in this work also features temperature control (**Figure 3**). The oxygen uptake rate (OUR) was calculated as follows: $OUR = V[CO_{2,inflow} - CO_{2,outflow}]$/liver weight [13], where V is culture medium flow rate (ml/min), CO_2 is the oxygen concentration (mg/l) in the medium. The inflow concentrations of dissolved oxygen in the hepatic artery versus the portal vein were measured separately. The outflow concentration of the hepatic

vein was also monitored together with the flow rate. Oxygen consumption was normalized according to the liver weight.

Ex situ liver culture

After 30 minutes of warm ischemia following 30 minutes of cold perfusion, rat livers were connected to the ESLC system at two sites: the artery was connected to a higher pressure tube (50 cmH_2O) and the portal vein was connected to a lower pressure tube (10 cmH_2O) as measured by gravity [10, 11]. The whole ESLC system was kept at 38.5°C. Since sinusoidal dilatation could be unintentionally induced [11], which may cause liver injury [14, 15], we reduced the flow from the portal vein so that the hepatic arterial flow and the portal venous flow were maintained at 40% (3 ml/min) and 60% (5 ml/min) of the total flow, respectively. The pressures for both the portal vein and the hepatic artery were maintained constantly. A diagram of the ESLC system and the actual system are shown in **Figure 2**.

BrdU proliferation assay

Bromodeoxyuridine (BrdU), a thymidine analogue, was incorporated into DNA in proliferating cells [10, 16, 17]. This BrdU assay has been utilized extensively to assess newly synthesized DNA in dividing cells [17, 18]. BrdU was used in the ESLC system at 100 M. BrdU positive cells were detected by immunohistochemistry following a standard protocol [18, 19]. The reagents used in this assay were purchased from the following sources: BrdU, from MedChem Express (Catalog No. HY-15910, Monmouth, NJ, USA); mouse anti-BrdU antibody, from Biolegend (Catalog No. 339802, San Diego, CA, USA); and secondary antibody against mouse IgG1, from Biogenex (Catalog No. QD400-60K, Fremont, CA, USA).

Histological assessment of biliary epithelial cells

Biopsies of extrahepatic bile ducts were taken at 6 hour after the start of ESLC. The samples were stored in formalin overnight and then exchanged for 75% ethanol on the next day till paraffin embedding. Paraffin-embedded sections were performed at 4 μm of thickness and prepared for hematoxylin and eosin (H&E) staining. Images were captured using Amscope (Catalog No. IN480TC-FL-BWF, CA, USA).

Statistical analysis

GraphPad Prism software 6 (GraphPad Software, Inc., La Jolla, CA, USA) was utilized for data analysis and ImageJ [11, 18, 20, 21] was used to quantify the area of sinusoidal spaces of liver from both groups and the areas of nucleus of biliary epithelial cells. Data are expressed as mean ± SD and analyzed with Student's t test.

Results

Oxygen consumptions of the liver at different temperatures

Currently preservation solutions in liver transplantation typically do not contain excess oxygen. On the other hand, several studies have indicated that the liver does consume oxygen and it also requires oxygen to maintain its function [10, 22, 23]. However, it was unclear how much oxygen the liver consumes at a low temperature. In this work, we have tested oxygen consumption of the rat liver at temperatures lower than 37°C. A diagram of oxygen consumption of the rat liver at different temperatures is shown in **Figure 3**. In this experiment, twenty rat livers were freshly isolated without warm ischemia and quickly connected to the perfusion system following a standard procedure [10, 11]. The perfusate was constantly oxygenated and the concentration of dissolved oxygen was constantly monitored in the inflows of the hepatic artery and the portal vein, and the outflow of the hepatic vein at given temperatures (**Figure 3B**). Considering oxygen evaporation during the procedure, the temperature controls were set in parallel for both the hepatic artery and the portal vein (**Figure 3B**). All data collected were within two hours in order to minimize influence of other factors on oxygen consumption measurement. Our results indicate that all livers tested so far consumed oxygen even at low temperatures. Moreover, the livers tended to consume more oxygen at higher temperatures (**Figure 3A**).

Impact of oxygen supply on liver morphology

Histological assessment of H&E stained biopsies of liver showed no major morphological differences in hepatocytes and biliary epithelial cells between the control and the experimental groups. For example, after 6 hours of ESCL we did not observe any significant difference in

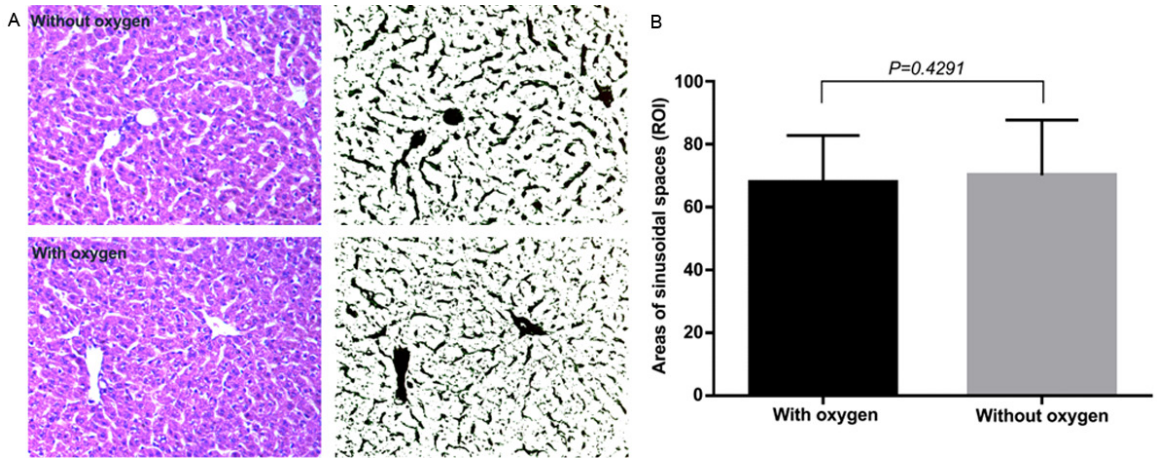

Figure 4. Morphologies of livers and biliary ducts with or without excess oxygen supply during liver isolation. A. Sinusoidal spaces from two groups. Top: sample from the control group, without oxygen supply; Bottom: livers were isolated with oxygen supply. Left panels show H&E staining and the right ones indicate that the sinusoidal spaces analyzed by ImageJ. B. Quantification of the morphologies. No significant differences were observed between the two groups.

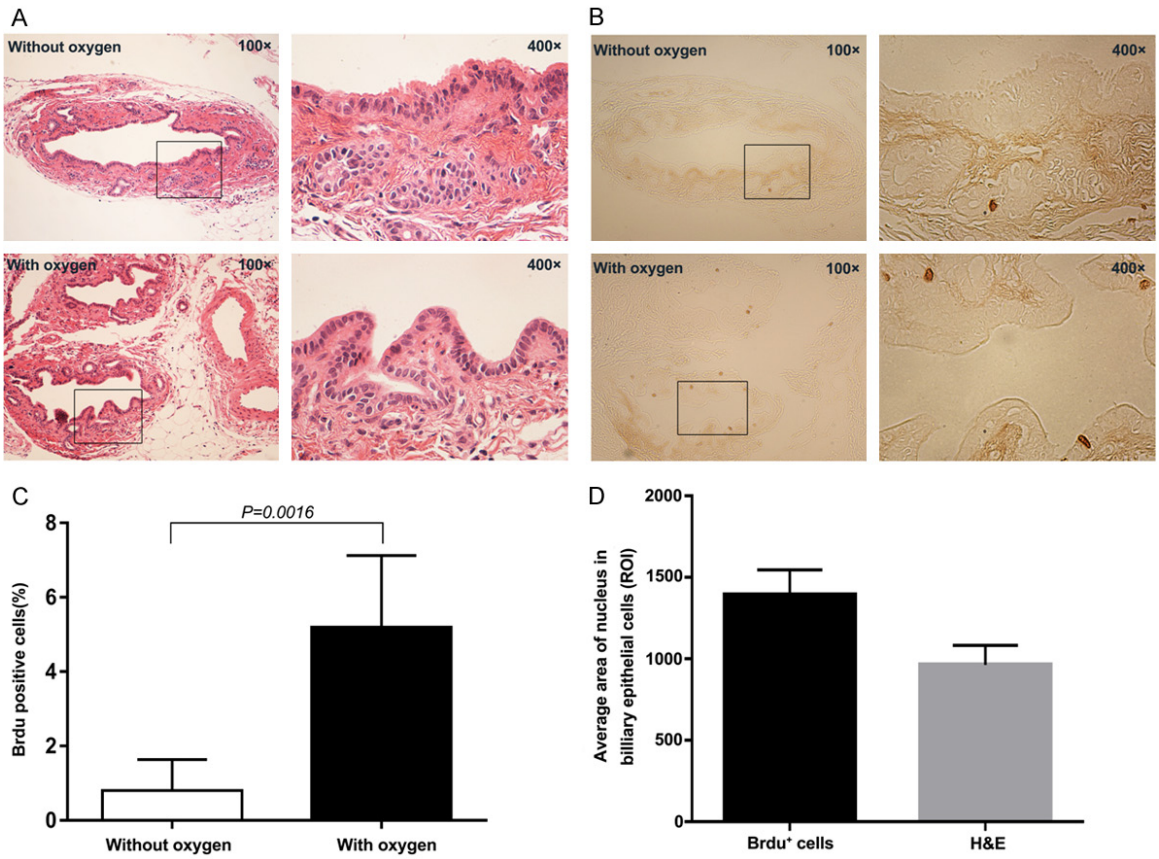

Figure 5. Biliary epithelial cells proliferated during 6 hours of ESLC. A. Images of H&E staining of bile ducts from two groups (top panels: without oxygen supply; bottom panels: with oxygen supply). Enlarged views are shown on the right panels. Only a slight difference was found in the mucosal lining in the control group without oxygen supply (arrow). B. BrdU staining in biliary sections. Left panels: sections of the biliary duct from the two groups. Enlarged views are shown on the right panels. Only one BrdU positive cell was detected in the peribiliary gland in the control group (top, without oxygen supply). In the experimental group (bottom), more than ten BrdU positive cells were detected

in the biliary duct with oxygen supply. In the enlarged view of the experimental samples, four of BrdU positive cells were found in the biliary duct. Among them, three were located in the peribiliary gland. C. Quantification of BrdU positive cells. D. Average area of nuclei in biliary epithelial cells. The *P* value is shown on the top of columns. Data are represented as means ± SEM (n = 6 independent experiments; *P<0.05).

sinusoidal spaces and morphology of hepatocytes between the two groups (**Figure 4A** and **4B**) [9]. Since integrity of bile duct and peribiliary glands are important for liver regeneration [22, 23], we also examined histological morphology of biopsies in extrahepatic bile ducts. Consistent with the work described previously [24], we did not observe any significant differences in mucosal lining and morphologies in the sub-epithelial stroma and peribiliary glands between the two groups (**Figure 5A**).

Proliferation of biliary epithetical cells in ESLC

We used BrdU assay to detect proliferating cells during ESLC. Biopsies of cultured livers were sectioned and processed. BrdU incorporated in proliferating cells were detected by the anti-BrdU antibody [9]. We found that from the livers that were supplied with oxygen during isolation approximately 4.5% of biliary epithelial cells were BrdU-positive, while almost none was found in the entire section in the control livers (no oxygen supply during liver isolation) (**Figure 5B** and **5C**). These results indicate that in the presence of sufficient oxygen biliary epithelial cells were proliferating during ESLC and oxygen supply during liver isolation was necessary for biliary epithelial cells to proliferate. The area of the nuclei in dividing biliary epithelial cells as detected by BrdU appeared larger than the average area of the nuclei identified by H&E staining [11, 18, 20, 21]. Clearly, without oxygen supply during liver isolation biliary epithelial cells lost their capability of proliferation in ESLC (**Figure 5D**). Consistent with this view, a recent study has also shown that oxygenated machine perfusion in rat liver model provides better preservation of biliary epithelial function and morphology [25]. Taken together, our data support that oxygen supply during liver isolation is necessary for biliary epithetical cells to proliferate in liver organ culture.

Discussion

In this study, we measured the amount of oxygen consumed by the rat *ex situ* livers at different temperatures. Our results indicate that livers tended to consume more oxygen at a higher temperature than at a lower one. Nevertheless, it should be noted that the oxygen consumption was quite variable among livers, and some livers still consumed a significant amount of oxygen even at a low temperature. It is possible that variability arises because livers we used were in different metabolic statuses due to differences in food intake prior to experiments. Other causes are equally possible. At this stage, we are unable to pinpoint what causes the variability in oxygen consumption among livers.

It is known that maintenance of the liver requires oxygen all the time after it is isolated from the donor [23, 26, 27] and that oxygenated perfusion reduces arteriolonecrosis of the peribiliary vascular plexus in preserved livers [22, 23]. However, it was unclear whether oxygen supply during liver isolation has any impact on liver function. In this study, we have addressed this question. Our study demonstrates that after 30 minutes warm ischemia oxygen supply during liver isolation is required for biliary epithelial cell proliferation. Biliary epithelial cells in the livers isolated after completing 30 min of warm ischemia following 30 min cold perfusion with a sufficient oxygen supply were able to proliferate in ESLC. In contrast, biliary epithelial cells in the control livers that were subject to 30 min cold perfusion without excess oxygen supply were unable to proliferate in ESLC. Consistent with previous findings [10], we found that BrdU could be detected as early as six-hour after the onset of liver culture.

In addition, we found that after six hours in culture hepatocytes proliferated more vigorously than biliary epithelial cells based on the BrdU assay. BrdU positive hepatocytes were frequently found side by side, suggesting they were daughter cells and mitosis was complete. The number of BrdU positive hepatocytes was similar to that observed in porcine livers [10]. In contrast, very rarely BrdU positive cells were found side by side in biliary epithelial cells, indicating these cells had not undergone mitosis and the cell cycle was incomplete. It is possible that biliary epithelial cells are subject to more stringent cell cycle regulation than the hepatocytes during ESLC. Whether these BrdU positive proliferating cells are also Sca-1 positive

[28] or p38 MAPK positive [29], at this stage, we do not have any additional evidence to prove or disprove it yet.

In conclusion, our data indicate that livers consume oxygen even at a low temperature and oxygen should be present in the entire procedure including liver isolation. The hepatocytes and the biliary epithelial cells are able to proliferate after 30 minutes of warm ischemia. Our results highlight the important role of oxygen from the liver preservation solution in maintaining the integrity of livers for regeneration *ex vivo* and to minimize the risk of biliary complications such as nonanastomotic biliary strictures after liver transplantation.

Disclosure of conflict of interest

None.

Address correspondence to: Qiru Xiong, Department of Chirurgery, First Affiliated Hospital, Anhui Medical University, 218 Jixi Road, Hefei 230022, Anhui, P. R. China. E-mail: xiongqiru2012@126.com; Lili Xu, School of Basic Medical Science, Anhui Medical University, 81 Meishan Road, Hefei 230032, Anhui, People's Republic of China or Department of Cell Biology, SUNY Downstate Medical Center, 760 Parkside Avenue, Brooklyn, New York 11226, USA. E-mail: Lili.Xu@downstate.edu

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