Isolation and short term cultivation of swine hepatocytes for bioartificial liver support system

Jun Li, Lan-Juan Li, Hong-Cui Chao, Qian Yang, Xiao-Li Liu, Ji-Fang Sheng, Hai-Ying Yu and Jian-Rong Huang

Hangzhou, China

BACKGROUND: **The demand for the clinical use of hepatocytes is increasing. The aim of this study was to develop a method for procurement of high qualitative pig hepatocytes and to evaluate the state of freshly isolated and cultured hepatocytes.**

METHODS: **The domestic extracorporeal circulating perfusion apparatus was used to isolate and harvest swine hepatocytes by the two-step perfusion method with EDTA and collagenase. The viability, function and morphology of the freshly isolated and cultured cells were evaluated and observed by the trypan blue exclusion test, biochemical measurements, phase contrast microscopy and transmission electron micrography (TEM).**

RESULTS: **The total yield of isolated hepatocytes reached to $1.5(\pm 0.4)\times 10^{10}$ per liver with a viability of 92(±5)%, and the purity of hepatocytes reached to 98%. Immediately after isolation, phase-contrast microscope and TEM showed that undamaged hepatocytes appeared bright, translucent and spherical in shape, with a characteristic well-contrasted border. After 24 hours, the concentrations of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), albumin (ALB), creatinine (Cr) and blood urea nitrogen (BUN) in the fluid of culture were declined significantly.**

CONCLUSION: **This method of procuring swine hepatocytes could get high quality cells with active metabolic function.**

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KEY WORDS: **bioartificial liver support system; porcine hepatocytes; cell isolation; cell transplantation**

Introduction

Since isolated intact rat hepatocytes were used to study liver cell physiology in 1968,[1] the cell procurement technique has made much progress and proven to be a significantly powerful and versatile system to analyze many hepatic functions.[2-5] Recently, a renewed interest in liver cell isolation and culture techniques has been generated by advancements in gene engineering therapy, by the development of the hepatocyte-based artificial liver support system,[6,7] and by cell transplant for the treatment of acute or chronic liver failure[8-10] and correction of genetic disorders resulted in metabolically deficient states.[11] Therefore, it is very important to develop techniques of isolation and purification of hepatocytes.

The purpose of this study was to find a good method for isolation of high qualitative swine hepatocytes and evaluate the yield, viability, morphology and function of freshly isolated and cultured hepatocytes with the trypan blue exclusion test, phase-contrast microscopy, transmission electron micrography (TEM), and biochemical analysis.

Methods

Reagents

The following reagents were purchased: collagenase IV (lot: 33058), heparin (lot: 20045) and sodium pentobarbital (lot: 55024) from Sigma Chemical Company (St. Louis, USA); William's E medium (cat: 1085439) and hepatoZYME-SFM (cat: 1088804) from Gibco BRL Life Technologies (USA); ethylenediamine-tetra-acetic acid (EDTA, cat: 12096), trypan blue (cat: 63541) from Life Technologies (Shanghai, China); cell culture flasks and plates from Nunc Medos

Author Affiliations: Key Laboratory of Infectious Disease, Ministry of Public Health of China; Institute of Infectious Disease, Department of Infectious Diseases, First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou 310003, China (Li J, Li LJ, Chao HC, Yang Q, Liu XL, Sheng JF, Yu HY and Huang JR)

Corresponding Author: Lan-Juan Li, MD, Department of Infectious Diseases, The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou 310003, China (Tel: 86-571-87236759; Fax: 86-571-87236755; Email: lilj@zjwst.gov.cn)

Company (Sydney, Australia).

Preparation of primary porcine hepatocytes

All animal experimental protocols were approved by the Animal Care Ethics Committee, Zhejiang University, China. Chinese experimental mini-pigs (CEMP) weighing between 6-10 kg were purchased from China Agricultural University, Beijing, China. The animals were initially anesthetized with sodium pentobarbital (40 mg/kg) and injected with oto-vein with heparin (15 IU/kg). Surgery was performed under strict aseptic conditions. Briefly, the abdomen of the pig was entered through a midline incision, the hepato-duodenal ligament was dissected and its structures were ligated and divided except for the portal vein. Then the liver was removed and placed in a sterile stainless steel bowl. The portal vein was cannulated with a silicon tube by *in vitro* sequential perfusion with 2 mol pre-warmed (38 ℃) EDTA solution (prepared with D-Hanks buf-fered salt solution, D-Hanks) at a flow rate of 180 ml/min for 5 minutes, continued 5 minutes at 120 ml/min through the domestic extracorporeal circulating perfusion apparatus. And then 0.05% collagenase IV solution prepared in Ca^{++}-enriched Hanks buffered salt solution (HBSS) at 38 ℃ was perfused for additional 10 minutes at a flow rate of 80 ml/min. The collagenase IV solution could be recirculated through the silicon tube by the extracorporeal circulating perfusion apparatus. After the liver capsule was detached from the parenchyma while stopping perfusion collagenase, Williams'E medium 150 ml was perfused (at 4 ℃) at a flow rate of 150 ml/min for one minute to terminate digestion. The liver was removed to another sterile bowl, the capsule was disrupted, and the digested liver parenchyma was filtered through a series of 150 μm, 80 μm pore stainless steel meshes. Cells were then washed with cold Williams'E medium and centrifuged at 4 ℃ three times at 120 ×g for 5 minutes. The hepatocytes were collected and suspended with Williams'E medium (4 ℃).

Assessment of hepatocyte yield, cell viability, function and morphology

The total yield of hepatocytes was assessed by a blood cell counting chamber under a light microscope. Cellular viabilities were estimated by 0.4% trypan blue exclusion. The concentrations of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), albumin (ALB), creatinine (Cr) and blood urea nitrogen (BUN) in the freshly isolated cell suspension and in medium were detected at 24h, 48h, 72h, 96h, 120h and 168h after culture respectively using a standard computerized autoanalyzer system. The morphology and the structure of the freshly isolated hepatocytes and cultured cells after 24 hours were observed by phase-contrast microscopy (approximately ×200 magnification) and TEM.[12]

Hepatocyte culture

Immediately after isolation, the hepatocytes were incubated at 37 ℃ in cell culture flasks with hepatoZYME-SFM medium at a concentration of 1×10^5/ml for 168 hours. Meanwhile the cell viability of the freshly isolated hepatocytes which were preserved with William's E medium at 4 ℃ for 24 hours was observed using a hemocytometer after staining with 0.4% trypan blue.

Statistical analysis

Mean values were expressed as mean ± standard deviation. The data were analyzed using SPSS10.0 software. Statistical significance was calculated by Student's *t* test or analysis of variation.

Results

Cell yield and viability

The freshly isolated hepatocytes were harvested using the domestic extracorporeal circulating perfusion apparatus by two-step perfusion with EDTA and a collagenase. The total cell yield reached $1.5(\pm0.4)\times10^{10}$ per liver and cellular purity of about 99%, after centrifugation at 4 ℃, 120 × g, for 5 minutes three times. Phase-contrast microscopy revealed that cell fragments and contaminating non-parenchyma cells such as endothelial cells, Kupffer cells, Itos cells or pit cells were less than 0.1%-0.5%. The viability of the freshly isolated hepatocytes was 92 (± 5)%. The highest cell viability was 98% shown by the trypan blue exclusion test. After preservation with William's E medium at 4 ℃ for 24 hours, the cell viability could rise to 95(±3)%.

Cell configuration

Light microscopic examination of the freshly isolated suspension provides information on the quality of the hepatocyte preparation. Phase-contrast microscopy showed that intact hepatocytes were translucent, bright and spherical in shape, with a characteristic well-contrasted border. Damaged cells appeared vacuoles in the cytoplasm, blebbing of the plasma membrane and swelling of the mitochondria (Fig. 1). The hepatocytes sticked equally to culture flasks for 24-hour culture, and those formed small cell clusters like grapes with oblate, polygonal configuration (Fig. 2). On TEM, immediately after isolation, the single hepatocyte losed its typical shape and all junction complexes. The typical bile canaliculi and sinusoidal complexes became vague, and short microvilli were scattered over the plasma membrane surface (Fig. 3). After 24-hour culture, they reaggregated into clusters, reestablished conjunction between cells and restructured polarity (Fig. 4).

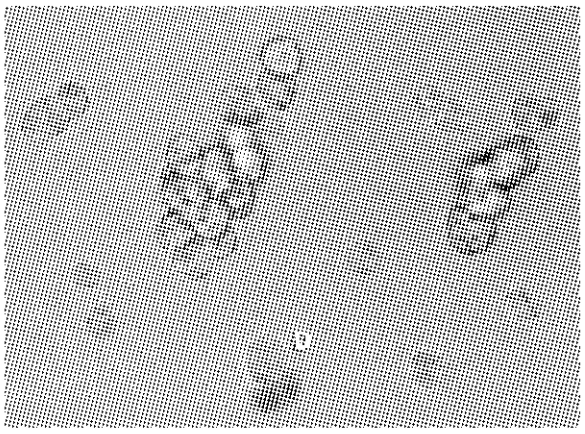

Fig. 1. Light micrograph of freshly isolated hepatocytes. Intact hepatocytes showing a translucent, bright and spherical shape, with a characteristic well-contrasted border. A damaged (D) cell taken up the dye and stained blue (original magnification× 200).

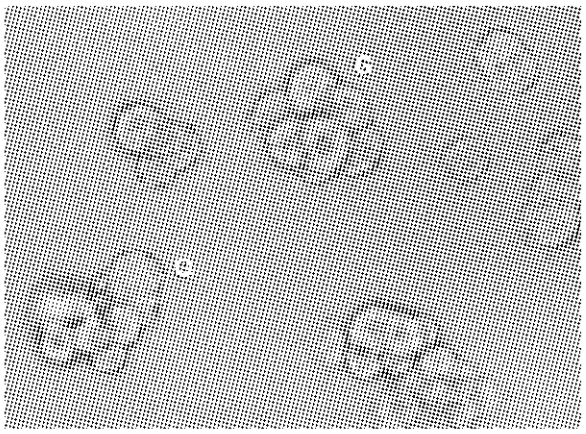

Fig. 2. Light microscopy micrograph of hepatocytes after 24-hour culture. The hepatocytes formed small cell clusters like grapes(G) with oblate, polygonal configuration (original magnification × 200).

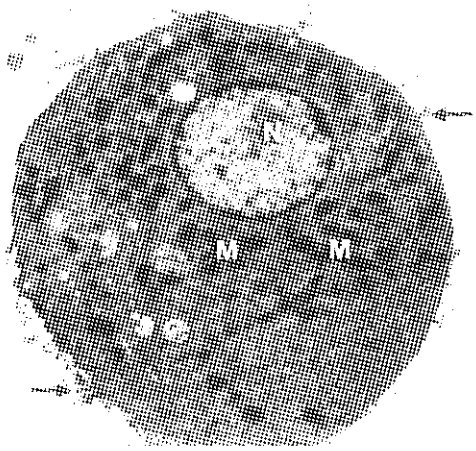

Fig. 3. Transmission electron micrograph of the freshly isolated hepatocyte. Intact nucleus (N) and mitochondria(M) in the cytoplasm are indicated. Typical bile canalicular and sinusoidal complexes become vague and short microvilli(short arrow) are scattled over the plasma membrane surface (original magnification×6000).

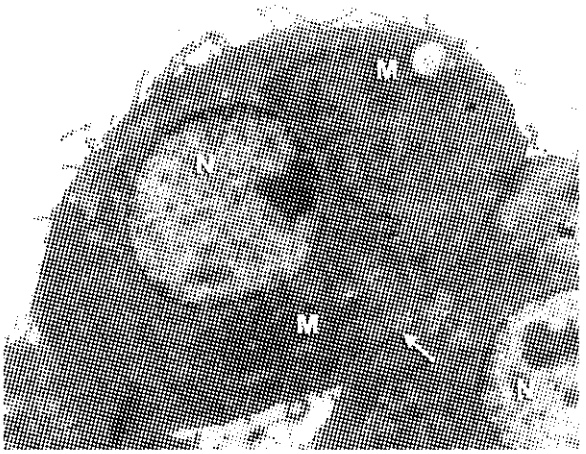

Fig. 4. Transmission electron micrograph of hepatocytes after 24-hour culture. They reaggregated into clusters and re-established bile canalicular junction (solid arrow) between cells and restructured polarity. Intact nucleus(N) and mitochondria(M) in the cytoplasm are displayed (original magnification×6000).

Table. The concentrations of ALT, AST, LDH, ALB, Cr and BUN in the culture medium and freshly isolated hepatocytes suspension

Hour	ALT(U/L)	AST(U/L)	LDH(U/L)	ALB(g/L)	Cr(μmol/L)	BUN(mmol/L)
Fresh suspension	86.6±112.3*☆	537.6±302.6☆	201.9±112.0*	2.27±1.22☆	19.3±4.0	0.76±0.40
24	9.8±21.3	209.1±220.0	96.9±72.3	1.13±0.43	21.3±7.6	0.78±0.52
48	1.7±3.2	55.1±68.8	43.6±53.3	1.30±0.13	26.0±3.2	0.35±0.11
72	0.67±0.58	12.7±10.6	25.7±13.0	1.53±0.40	21.0±1.0	0.76±0.86
168	0.50±0.71	33.0±1.4	24.5±3.5	0.95±0.07	17.0±1.4	0.17±0.02

☆: Compared with 24h, 48h, 72h and 168h groups, $P<0.01$; *: compared with 24h group, $P<0.05$ and compared with other group, $P<0.01$; △: compared with 24h group, $P<0.01$.

Cell function

The levels of ALT, AST and LDH in the medium (after 24-hour culture) were decreased obviously ($P<0.01$) while those of ALB, Cr, and BUN were not changed ($P>0.05$) as compared with the freshly isolated hepatocytes suspension (Table).

Discussion

Isolated hepatocytes which could maintain metabolic function for several hours in cells[13] are sensitive to hormones.[14] In recent years, attention has been attracted to use primary cultured hepatocytes to establish a

bioartificial liver support system (BALS)[15-17] and perform cell transplantation to replace hepatic functions in patients with acute or chronic liver failure.[18,19] However these methods with hepatocytes from humans have been limited by the shortage of normal donor liver.[20,21] Many studies have demonstrated that pig hepatocytes have several advantages over other potential donor species for clinical use, including unlimited availability, breeding characteristics, and physiological and immunological similarities to humans.[22] It could be a suitable hepatocyte source for BALS.[23] The clinical and animal experiments of Nelson and Samuel using isolated porcine hepatocyte to construct BALS to treat fulminant hepatic failure (FHF) models and patients have shown satisfactory results.[24]

Since Howard and Pesch isolated intact rat hepatocytes for the study of liver cell physiology, the techniques of isolating hepatocytes have been greatly improved.[25] We successfully used Seglen's two-step liver perfusion method to isolate rat hepatocyte but failed to isolate porcine hepatocyte. We improved the two-step method by the extracorporeal circulating perfusion apparatus with EDTA and collagenase IV solution to prepare pig hepatocyte suspension. The cell yield reached to 1.9×10^{10} per liver and the cell viability approached to 97%, as assessed by the trypan blue exclusion test. According to our experiences, there are many keys in the procedure of digestion and isolation. First, the increase of the concentration of calcium can enhance the activity of collagenase. Second, the temperature of collagenase solution at 38 ℃ is suitable. If it is too low, the activity of collagenase may be influenced and the viability of hepatocytes decreased. Third, using Williams'E medium to terminate digesting and washing could decrease the damage cell member and raise the viability of isolated hepatocyte. Last, cell centrifugation is also very significant, because it is usually difficult to remove thoroughly cell fragment and non-parenchyma cell. We decreased the rate of contamination of non-parenchyma to $0.8(\pm0.3)$% after centrifugation, $120\times g$, at 4 ℃ for 5 minutes three times. The configuration and form of cells were well protected, and their viability rose comparatively as shown by the phase-contrast microscope and transmission electron microscope.

We found that the concentrations of ALT, AST and LDH in the freshly isolated hepatocyte suspension were relatively higher than those after 24-hour culture and lower after 48 hours.

In conclusion, the freshly isolated hepatocytes after 24-hour culture are best not only in morphology but also in function. Thus it is the best choice to use them in BALS for treatment. A new pathway using isolated pig hepatocyte to treat patients with acute or chronic liver failure has been available in our laboratory. We are sure that the liver genetic disorders that result in metabolically deficient states will be corrected in future with the developing of cell molecular biology, genetic engineering, and techniques of cell culture and transplantation.[25,26]

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Contributors: LJ proposed the study, completed most experiments and wrote the first draft. LLJ proposed the study and critically read the manuscript. CHC completed part of the experiment and analyzed the data. LXL completed part of the experiment. SJF contributed to data analysis. YHY and HJR completed part of the experiment. All authors contributed to the design and interpretation of the study and to further drafts. LLJ is the guarantor.
Competing interest: No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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