An improved experimental method for simultaneously isolating hepatocytes and hepatic stellate cells in mouse liver infected with *Echinococcus multilocularis*

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**Abstract**

**Background:** Alveolar echinococcosis (AE) is a zoonotic disease caused by the larval stage of *Echinococcus multilocularis* parasitizing in the human liver, causing local pathological changes in the liver and manifesting as hyperplasia, liver fibrosis, atrophy, degeneration, and necrosis. Here, we report a method that can simultaneously isolate hepatocytes and hepatic stellate cells (HSCs) from mice infected with *Echinococcus multilocularis*.

**Methods:** A mouse model of AE was established. Hepatocytes and HSCs were isolated from mouse liver using a two-step method combining in situ collagenase perfusion and gradient centrifugation. Expressions of Alb, Desmin, and α-SMA were detected with immunofluorescence to identify the isolated hepatocytes and HSCs.

**Results:** The viability and purity of hepatocytes and HSCs both reached 90% or above. For hepatocytes, clear cell boundaries were observed, and the nuclei were round or oval, with clear nucleoli. There was a homogeneous distribution of the hepatocyte marker Alb in the cytoplasm of hepatocytes. Lipid droplets and Desmin expression were observed in the cytoplasm of freshly isolated HSCs. During the activation of HSCs, the lipid droplets gradually decreased and disappeared with a high expression of α-SMA.

**Conclusion:** Hepatocytes and HSCs are simultaneously isolated. This may provide a research tool to investigate the interaction between hepatocytes and HSCs and to investigate the mechanism of *Echinococcus multilocularis* infection-induced liver pathological changes.

**Keywords**

*Echinococcus multilocularis*, hepatic stellate cells, hepatocytes, in situ collagenase perfusion, simultaneously isolation

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1 | INTRODUCTION

Alveolar echinococcosis (AE) is a zoonotic disease caused by the larval stage of *Echinococcus multilocularis* (EM) parasitizing in the human liver, and it brings serious harm to the health of the host. Infection of EM causes local pathological changes in liver, manifesting as hyperplasia, liver fibrosis, atrophy, degeneration, and necrosis. The lesion edges present invasive spread (cancer-like buds). \(^1\)\(^2\) Reducing liver injury or reversing liver fibrosis has become the focus of recent research on AE. Liver fibrosis is a complex pathological process involving a variety of liver cells and cytokines. \(^3\) Hepatocyte apoptosis and hepatic stellate cell (HSC) activation are suggested to be closely associated with liver fibrosis, as apoptosis of hepatocytes significantly increases in liver fibrosis, accompanied by poor cell proliferation, HSC activation, and collagen fiber hyperplasia. \(^4\)\(^6\)

Apoptosis is the first response of hepatocytes to toxins under stress. These apoptotic hepatocytes release lipid signals that can be received by HSCs. Subsequently, HSCs engulf apoptotic bodies and release the fibrotic factors to further activate the HSCs. This persistent HSC activation will further promote hepatocellular apoptosis, leading to the peak of hepatic inflammation. \(^7\)\(^8\)

The animal model is important in investigating the liver pathogenesis caused by the infection of EM. Herein, we established a method of simultaneously isolating hepatocytes and HSCs from livers of mouse models with AE. This will provide a research tool to further investigate the interaction between these two cells and provide valuable information on fibrosis caused by infection of EM.

2 | MATERIALS AND METHODS

2.1 | Animals

Female C57BL/6 mice aged 8 weeks or above and weighing about 25–30 g were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (License No. SCXK (2012–0001). The mice were maintained in the animal center of the First Affiliated Hospital of Xinjiang Medical University, housed in an individually ventilated cage at 24°C, and were with free access to water and food under 12 h-light/12 h-dark cycle. All experiments involving animals were approved by the Ethical Committee of the First Affiliated Hospital of Xinjiang Medical University (approval no. 20140411-05).

2.2 | Mouse model of AE

After 7 days of routine feeding, the AE model was established. Briefly, mice were anesthetized with Zoletil (60 mg/kg, Virbac S.A) intraperitoneally. A 2 cm incision was made on the abdomen and the portal vein was exposed. The EM suspension of 2000/200 μl (from the Department of Insects, Animal Center of the First Affiliated Hospital of Xinjiang Medical University) was injected into the mouse portal vein. The incision was closed with a suture after hemostasis. The AE model mice were fed for 3 months before the liver cell isolation.

2.3 | Two-step in situ perfusion of the liver

The C57BL/6 mice infected with EM were sacrificed with CO2 inhalation. In situ perfusion of the liver was performed. Briefly, the liver was first perfused with 0.5 mmol/L EDTA in D-Hanks via the distal end of the portal vein with a peristaltic pump. The air in the soft hose of perfusion bump was completely exhausted before perfusion. The color of the liver turned white after about 3 s, and the procedure continued for about 2 min. Then, the liver was perfused with 0.5 mg/ml Protease E in D-Hanks for 2 min followed with 200 U/ml-type I collagenase in D-Hanks for 5 min. The flow rate was adjusted to 3.0–4.0 ml/min. Perfusion was terminated when the liver became soft and there was no rebound after pressing.

2.4 | Hepatocyte isolation and culture

After perfusion, the mouse liver was removed and incubated with 20 ml of high glucose DMEM containing 200 μ/ml type I collagenase and 0.5 mg/ml protease E. The obtained cell suspension was filtered with a 70-μm sterile cell filter and centrifuged for 2 min twice. The supernatant was the non-parenchymal cell layer and it was collected. The cells obtained by centrifugation were washed twice with D-Hanks solution, centrifuged at 45 × g for 2 min, and then resuspended. After mixing the hepatocyte suspension 1: 1 with Trypan Blue, 10 μl of the mixture was added dropwise to the blood cell count plate to detect hepatocyte survival rate. Hepatocytes were seeded in a six-well culture plate (Corning) pre-coated with type I collagen at 1 × 10^4/ml, or were seeded onto glass coverslips pre-coated with type I collagen. They were cultured in medium and maintained at 37°C with 5% CO2. The medium was changed after 4–8 h and then changed every 2 days.

2.5 | Hepatic stellate cells isolation and culture

The non-parenchymal cell-enriched supernatant was collected and centrifuged at 4°C, 580 × g for 10 min. The precipitate was resuspended to 8 ml with GBSS-B, and 4 ml of Nycodenz (Axis-Shield) was added and mixed. The mixture was slowly added with 2 ml of GBSS-B, and centrifuged at 4°C, 1830 × g for 17 min. After centrifugation, the hepatocytes precipitated at the bottom of the tube, and the HSCs formed a white annular layer in the middle. The intermediate layer containing HSCs was carefully collected, washed with GBSS-B, and then centrifuged at 4°C, 580 × g for 10 min. The supernatant was discarded and the HSCs were resuspended with HSCs culture medium. HSCs were inoculated into petri dishes at a density of 5 × 10^5/ml. The medium was changed after 24 h and then changed every other day.
2.6 | Hepatocyte morphology and function

Hepatocyte morphological observation was carried out under an inverted phase-contrast microscope. The hepatocytes growing on glass coverslips were fixed with 4% paraformaldehyde and stained with Alb immunofluorescence using an anti-Alb antibody (Abcam). The cells were stained with DAPI and subjected to confocal observation.

2.7 | Identification of HSCs

The freshly isolated HSCs were observed under UV excitation at 328 nm. HSCs were cultured for 7 days and were fixed with 4% paraformaldehyde. Then, they were subjected to α-SMA and Desmin immunofluorescence staining using anti-α-SMA and anti-Desmin antibodies (Abcam). DAPI was used for nuclear staining.

3 | RESULTS

3.1 | Number of cells and survival rate

The hepatocytes were isolated by modified in situ perfusion and low-speed centrifugation (Figure 1). About $5.5 \times 10^7$ hepatocytes were obtained per mouse. About $5 \times 10^5$ HSCs were obtained per mouse using Nycodenz density gradient centrifugation. The viability and purity both reached 90% or above.

3.2 | Hepatocyte morphology

Cells started adherent growth after 4–6 h of culture and presented irregular paving stone-like morphology. Clear cell boundaries were observed under an inverted microscope. The nuclei were round or oval, with clear nucleoli. Some hepatocytes fused into island colonies, as shown in Figure 2.

3.3 | Hepatocyte marker A1b expression

Hepatocytes seeded on small glass slides were stained by Alb immunofluorescence after 48 h of culture. The staining showed a homogeneous distribution of the hepatocyte marker Alb in the cytoplasm of hepatocytes, as shown in Figure 3.

3.4 | Hepatic stellate cells morphology

Under the inverted microscope, there were lipid droplets in the cytoplasm of freshly isolated HSC with strong refraction. The HSCs were round and exhibited autofluorescence at the wavelength of 328 nm (Figure 4A). The diameter of HSCs was 10–15 μm, which was smaller than that of hepatocytes (25–40 μm). After 24 h of culture, most of the cells presented oval shape and adherent growth, and some cells began to have pseudopods (Figure 4B). After 2–3 days of culture, the cell adherence showed diversity. Cell growth showed spreading and some already had pseudopods, presenting typical morphology of HSCs. Cells could be passaged after 7–8 days of culture.

FIGURE 1 Flow chart of hepatocyte and hepatic stellate cell isolation. (A) Liver in situ perfusion. (B) Intrahepatic cell suspension and in vitro digestion. (C) Low-speed centrifugation for hepatocytes isolation. (D) Density gradient centrifugation for hepatic stellate cells isolation.
3.5 Hepatic stellate cell marker detection

Hepatic stellate cells were plated on glass coverslips and Desmin immunofluorescence staining was performed after 24 h of culture (Figure 5). Rich lipid droplets and Desmin expressions were observed. The α-SMA immunofluorescence staining was performed after 7 days of culture (Figure 6). During the activation of HSCs, the lipid droplets gradually decreased and disappeared. After 7 days of culture, HSCs were completely activated and become myofibroblast-like cells. Lipid droplets completely disappeared with a high expression of α-SMA (Figure 6).
DISCUSSION

Alveolar echinococcosis causes lesions that are similar to liver carcinoma, and it is known as worm-cancer because of its invasive proliferation. Liver fibrosis is one of the main pathological changes during AE. Apoptosis of the liver cells is not only caused by inflammation and fibrosis but also exacerbates these reactions, especially in the HSCs (the main cell type contributing to fibrosis). HSCs can engulf apoptotic bodies, stimulate the expression of TGF-β1 and induce the formation of type I collagen, leading to hepatic fibrosis. The hepatocyte apoptosis induced by infection of EM and the activation of HSCs are the focuses in the study of liver fibrosis mechanisms. It is urgent to establish a stable and efficient method for simultaneous isolation of hepatocytes and HSCs. However, the number of HSCs is small, and HSCs and hepatocytes are easy to adhere. The key novel point in this study is to efficiently isolate hepatocytes and HSCs simultaneously in mice infected with EM.

First, a perfusion method without pronase digestion was used in this study. The techniques of isolating hepatocytes or HSCs alone in the normal liver have been established and reported, whereas the simultaneous isolation of hepatocytes and HSCs from mice infected with EM has been rarely reported. Simultaneous isolation ensures that the two types of cells are from the same infected individual, and the two types of cells interacted under the same setting. In this study, we first used EDTA to perfuse the liver, and then used protease E and type I collagenase as perfusate to obtain cell suspension. The cell suspension was centrifuged at a low speed to obtain hepatocytes. The supernatant was non-parenchymal cells (including Kupffer cells, HSCs, hepatic endothelial cells, etc.). This perfusion method omitted the digestion with pronase, which not only shortened the operation time, but also reduced the damage of pronase to cells and ensured the purity of the isolated cells.

Then, we used 8%–8.2% Nycodenz as the isolation medium. The most commonly reported media for the isolation of primary HSCs...
include Nycodenz, Percoll, and Optiprep et al. In the pilot experiments of this study, Percoll, Optiprep, and Nycodenz were used. However, the purity of the isolated HSC was low and there were many impurities when we used Percoll and Optiprep. The purity was high when Nycodenz was used. Therefore, Nycodenz was used as the isolation medium in this study. Repeated experiments have demonstrated that the cell layer after gradient centrifugation is the most evident and the number of cells is the largest when the concentration of Nycodenz is at 9%–9.6%. In order to improve the purity of HSCs, Weiskirchen has established a flow sorting method. However, the viability of cells declined due to the shear force in this method, and it was also costly. Thus, we did not use this method in this study.

Third, we used a peristaltic pump instead of the traditional manual injection method to perfuse the liver. The infusion rate was uniform, thus reducing human errors during the operation, and improving the isolation efficiency. Finally, we used non-circulating in vivo perfusion. Compared with the method of in vitro perfusion of the liver, this method can not only improve cell purity, but also improve isolation efficiency. This method not only ensures high purity and high survival rate but also shortens the operation time within 1.5–2 h. This method is suitable for the simultaneous isolation of hepatocytes and HSCs in mice infected with EM. The obtained cells can be used for investigating the effect of EM on liver fibrosis or the interaction between hepatocytes and HSCs.

However, in the whole isolating process, attentions need to be paid to details, which will greatly impact the cell survival rate and the purity, including: 1) Mouse age and weight. We selected mouse aged 8 weeks or above and mouse weighted above 25 g. A greater number of isolated HSCs will be yielded from mice of older age; 2) air in the soft hose of perfusion bump needs to be completely exhausted before perfusion when using the peristaltic pump, otherwise, it leads to inadequate perfusion and declines in numbers of cells obtained; 3) when perfusion is completed, the liver connective tissues such as Glisson, liver ligament, and gall-bladder, etc., should be removed, thereby reducing the contamination from other cells. The time of liver digestion by collagenase should not be too long, otherwise, it will affect the cell viability; 4) when inoculating cells, the density of inoculated cells should be appropriate. A too high density will affect cell growth, while a too low density will weaken the interaction between cells and also affect cell growth; 5) In the culturing of liver cells, type I collagen was pre-coated to the culture plate to facilitate adherence of hepatocytes. Many factors were added into the culture medium to induce hepatocyte adherent growth.

5 | CONCLUSION

In summary, the method of simultaneously isolating hepatocytes and HSCs from mouse liver infected with EM has been established through improving the techniques of primary isolation and culturing. This method provides a research tool to further investigate the interaction between hepatocytes and HSCs, and is of great significance in the study of the EM infection-induced fibrosis.

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Not applicable.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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