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Liver Bioengineering Using Decellularized Whole-Liver Scaffolds

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Abstract

Currently, due to the progress made in the field of regenerative medicine, whole-organ bioengineering is becoming a valid alternative to cope with the shortages of organs for transplantation. In this chapter, we describe the main techniques carried out for pig liver bioengineering, which serves as an essential model for future human liver bioengineering. These include porcine whole-liver decellularization, endothelial and mesenchymal stem cell isolation, porcine ES-derived hepatoblasts, and scaffold recellularization using a bioreactor perfusion system.

Keywords Decellularization, Liver scaffold, Organ bioengineering, Perfusion bioreactor system, Recellularization

1 Introduction

Due to its role in the production of several proteins, vitamins, lipids, and carbohydrates, the synthesis of substances necessary for homeostasis and digestion, detoxification of various metabolites, and glucose level regulation, the liver is one of the body's vital organs in metabolism and homeostasis.

The high mortality of end-stage liver disease is a global public health issue, to which liver transplantation is the only definitive treatment to improve the survival and quality of life of these patients. However, there is still a significant gap between organ supply and demand.

Whole-organ bioengineering and regenerative medicine are promising new technologies that can help reduce liver shortage by increasing the number of organs available for transplantation. In this perspective, decellularization is an attractive technique in regenerative medicine to prepare scaffolds. This consist of the removal of cells from a tissue or organ using detergent perfusion through the vasculature, leaving behind an intact structure of the extracellular material, which is fundamental to regulate cell differentiation and function. The perfusion decellularization method represents a useful

procedure to create whole-organ scaffolds ready to be recellularized with freshly isolated cells and maintained in bioreactors for cell expansion, differentiation, and function [1, 2]. In this protocol, we describe how to prepare porcine liver scaffolds and isolate and expand endothelial, mesenchymal stem cells and iPS-derived hepatoblasts for posterior recellularization and maintenance of this bioengineered liver in a bioreactor perfusion system.

2 Materials

2.1 Liver Harvesting and Cannulation

1. Scalpel.
2. Forceps (with and without teeth).
3. Scissors.
4. Silk suture 4-0.
5. Cannula 18G.
6. Straight fitting 6.4 mm diameter.

2.2 Decellularization and Scaffold Preparation for Bioreactor

1. Distilled water.
2. Peristaltic pump with 14G and 17G tubing (Masterflex L/S with Masterflex L/S easy load pump head, Cole Parmer).
3. Silicone tubing (silicone tubing size 14G and 17G, Cole Parmer).
4. Pulse dampener.
5. Pressure sensor (APT Pressure Transduced, Panlab).
6. Pressure controller (Panlab).
7. Male Luer.
8. Four-way stopcock.
9. Detergent solution: 1% Triton X-100 with 0.1% ammonium hydroxide in distilled water.
10. X-ray source.
11. 150 cm culture dish.

2.3 Cell Isolation and Culture

1. Piglet umbilical cord.
2. PBS− (without calcium and magnesium).
3. PBS+ (with calcium and magnesium).
4. 0.05% trypsin/EDTA.
5. Neutralization medium: DMEM/F12 with 10% FBS.
6. 18G cannula.
7. Endothelial growth medium 2.
8. Fibronectin (5 μg/cm^2).

9. 6-multiwell cell culture plate.
10. 150 cm cell culture dish.
11. 50 mL conical centrifuge tubes.
12. Pig femur.
13. Surgical saw.
14. 18G needle.
15. Scalpel.
16. Scissors.
17. Forceps.
18. Bovine gelatin (0.02%).
19. 100 and 40 μm cell strainers.
20. Histopaque-1077.
21. Centrifuge.
22. Incubator at 37 °C and 5% CO_2.
23. MEF medium: 81% DMEM high glucose with 15% FBS, 2% P/S, 1% L-Glut, 1% DMEM nonessential amino acids.
24. Matrigel (Geltrex hESC-qualified reduced growth factor basement membrane) – diluted to 2 mg/mL in DMEM/F12 medium.
25. TrypLE.
26. 293FT cells.
27. Gelatin.
28. 100 mm tissue culture dish pre-coated with 0.1% gelatin.
29. DMEM high glucose.
30. 293FT medium: DMEM high glucose with 10% FBS.
31. FuGENE HD Transfection Reagent.
32. rtTA plasmid.
33. STEMCCA (OKSM) Lentivirus Reprogramming Kit.
34. D8.9/psPAX2 plasmid.
35. VSV-G plasmid.
36. Microcentrifuge.
37. Sterile microcentrifuge tubes (1.5 mL).
38. 0.45 μm filters.
39. Beckman Coulter Optima L-90K ultracentrifuge with SW-32 rotor.
40. 0.25% Trypsin-0.53 mM EDTA.

41. Polybrene.
42. Doxycycline.
43. Hemocytometer.
44. Pluripotent stem cell medium: mTeSR1.
45. RPMI cell differentiation medium: RPMI 1640, HEPES medium with 1% nonessential amino acids, 1% P/S.
46. B27 without insulin.
47. B27 with insulin.
48. Activin A.
49. Bone morphogenetic protein 4.
50. Fibroblast growth factor 2.
51. Hepatocyte growth factor.
52. EGM2 bullet kit (Lonza).
53. MCDB 131 culture medium (ThermoFisher Scientific).
54. DMEM/F12 culture medium (ThermoFisher Scientific).

2.4 Bioreactor System

1. Bioreactor vessel (Glass Ball Spinner, 250 mL, Bellco Biotechnology Inc., Vineland, NJ, USA).
2. Peristaltic pump (same description as in Sect. 2.2, item 2).
3. Silicone tubing (same description as in Sect. 2.2, item 3).
4. Pressure sensor (same description as in Sect. 2.2, item 5).
5. Pressure controller.
6. Magneto.
7. Magnetic stirrer.
8. Pulse dampeners (Cole Palmer).
9. Four-way stopcocks (Cole Palmer).
10. Smart site connections (Cole Parmer).
11. Luer-lock syringe 20 mL.
12. 20 μm filter.
13. Empty (sterile and clean) culture media bottle (as a secondary reservoir).
14. Bioreactor culture medium: 50% DMEM/F12 + 50% MCDB 131 containing 5% FBS, 1% L-glutamine, 1% penicillin/streptomycin, 5 μg/mL insulin, 10 μg/mL transferrin, 50 μg/mL VEGF, 40 μg/mL EGF, 40 μg/mL FGF-2, and 40 μg/mL IGF-1.

3 Methods

3.1 Pig Cadaveric Liver Harvesting

1. The suprahepatic vena cava is dissected as close to the atrium as possible.
2. The common bile duct is dissected as close to the duodenum as possible.
3. The portal vein is carefully dissected from the surrounding tissue, to visualize it and its branches. Lateral branches are ligated with 4-0 silk suture and cut as close to the intestines as possible.
4. The hepatic artery is carefully dissected from the surrounding tissue, to visualize it and its branches. Lateral branches are ligated with silk suture 4-0 and cut as close to the stomach as possible.
5. Infra-hepatic vena cava is carefully dissected and cut without damaging the lobe. The cut should be performed before the renal bifurcation (see Note 1).
6. Liver attachments are cut to remove the intact organ.
7. The diaphragm is carefully dissected around the esophagus.
8. The portal vein is cannulated with a straight fitting of 6, 4 mm diameter. The hepatic artery should be cannulated with a 20G cannula.
9. The gallbladder is emptied and cleaned.

3.2 Liver Decellularization

Decellularization is usually performed at room temperature, after one freezing/thawing cycle. Scaffolds are kept sterile at 4 °C until use. Components are assembled as shown in Fig. 1, and results are seen in Fig. 2.

1. Cannulas are attached to a peristaltic pump by using 17G tubing for the portal vein and 14G for the hepatic artery.
2. 2 L of distilled water is perfused through the portal vein at a pressure of 25 mmHg for both the hepatic artery and the portal vein.
3. 10 L of decellularization solution is perfused afterward (see Note 2).
4. 20 L of distilled water is perfused to remove the detergent from the tissue.

3.3 pUVEC Isolation

1. Umbilical cords are obtained from piglets that had undergone spontaneous abortions in farms of animal production.
2. Locate umbilical cord vein and inject 20 mL of PBS with a 18G cannula.

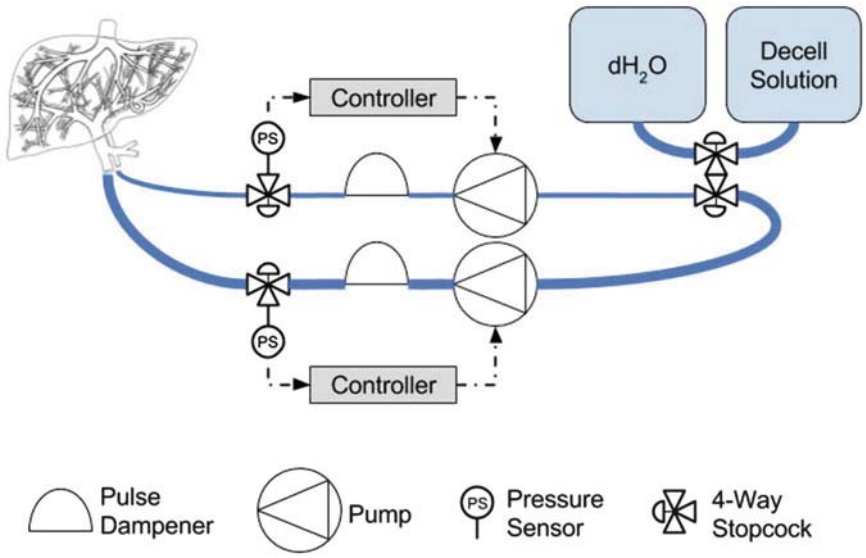

Fig. 1 Schematic diagram of the decellularization setup. The fine line represents the hepatic artery line; the thicker one represents the portal vein line. The liver is first washed with distilled water and then perfused with the decellularization solution detergent. Finally it is washed again to remove the detergent from the liver

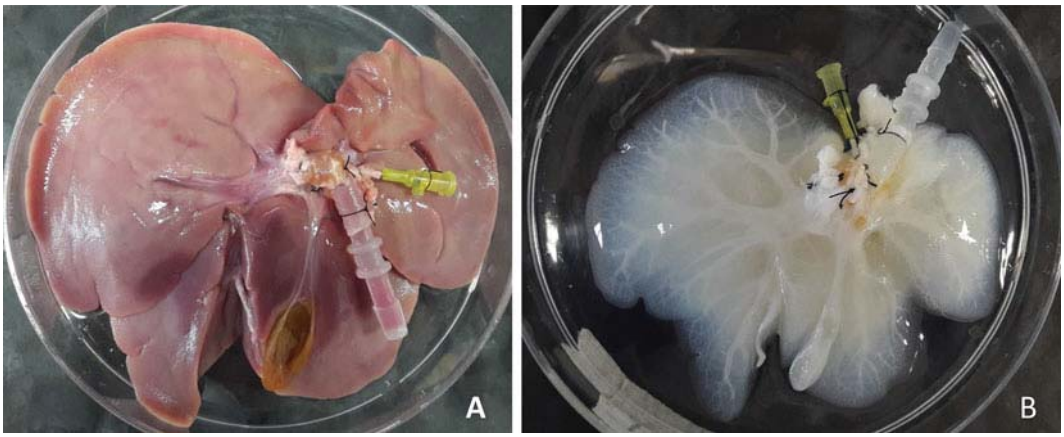

Fig. 2 Piglet liver before (**a**) and after decellularization (**b**)

3. Inject 20 mL of pre-warmed trypsin. Collect and inject again. Repeat this step for 7 min.
4. Collect all the trypsin and neutralize with 20 mL of DMEM with 10% FBS and centrifuge 400 × *g* for 5 min.
5. Aspirate the supernatant and resuspend the pellet in 10 mL of EGM-2 with 20% FBS.
6. Transfer the cells to a 6-multiwell dish coated with fibronectin (0.03 mg/mL), and place it into the incubator at 37 °C.
7. Allow the cells to grow for 3–4 days. Change media at day 4.

8. Once some colonies are detected, change media to EGM-2 with 10% FBS. Change media every 3 days.
9. Trypsinize the culture when it reaches 80% confluence (see Note 3).

3.4 pMSC Isolation

1. Pig bone marrows are isolated from cadaveric piglets slaughtered by farms of animal production due to malformations or trauma.
2. Femurs are carefully dissected and cleaned from muscle/ligament tissue.
3. Cut the epiphysis and flush the bone marrow with a 18G needle with 5 mL of DMEM and 1% P/S.
4. Collect this cell suspension and centrifuge at 300 × *g* for 5 min.
5. Reconstitute the pellet with 10 mL of DMEM +1% P/S, and filter sequentially through 100 and 40 μm cell strainers.
6. Separate with a histopaque density gradient centrifugation.
7. Resuspend the pellet with 10 mL of DMEM/F12 with 10% FBS and 1% P/S, and plate the cells in a 10 cm culture dish previously coated with a bovine gelatin solution (0.2%). Place it in the incubator.
8. Allow the cells to grow for 2 days. Change media at day 2.
9. Trypsinize the culture when it reaches 70–80% confluence.

3.5 Generation of iPS-Derived Hepatoblasts

Cultured porcine mesenchymal stem cells with less than four passages were reprogrammed to generate iPS cells by transduction with four human reprogramming factors: Sox2, Klf4, Oct4, and c-Myc. We followed the protocol published by Rajarajan et al [3]. Once the iPS cells were obtained and characterized properly, they were differentiated to hepatoblast-like cells.

3.5.1 Lentiviral Production

1. Culture 293FT cells onto gelatinized 100 mm dish in 293FT media until cells reach 80–90% confluency.
2. Mix in microcentrifuge tube 770 μL DMEM high glucose with 50 μL FuGENE Reagent per 10 cm dish, and incubate for 5 min at room temperature.
3. Add 5.5 μL VSV-G and 8.25 μL D8.9 and mix well.
4. Add a total of 11 μg vector DNA (STEMCCA Lentivirus Reprogramming Vector pluripotent transcription factors at 11 μg/infection). Mix gently and incubate for 30 min at room temperature.
5. During incubation, add 10 mL of fresh 293FT medium to 293FT cells.
6. Add entire FuGENE/DNA complex to the 100 mm dish drop by drop, and rotate the plate to mix the contents. Incubate for 12–24 h.

7. Change 293FT medium 12–24 h later.
8. After 24 h, collect all media and store at 4 °C. Add 10 mL of fresh 293FT medium to the dish. Repeat every subsequent 24 h for three collections.
9. Filter collected viral medium through a 0.45 μm filter, and centrifuge at 50,000 × *g* for 1.5 h at 4 °C.
10. Decant supernatant and add 200 μL of serum-free DMEM medium to the pellet and let it stand overnight at 4 °C.
11. Resuspend, aliquot, and store the virus at −80 °C.

3.5.2 Infection of Fibroblasts Using Pluripotency Transcription Factors

1. Culture pig MSC in a gelatin-coated single well of a 6-well plate until 90% confluency.
2. Trypsinize the cells in culture, count the number of cells, and adjust the cell concentration to 10^4 cells/mL.
3. Add polybrene at a final concentration of 8 μg/mL to the cell-containing medium. Mix by pipetting up and down.
4. Thaw the four viral constructs Oct-3/4, Sox2, Klf4, and c-Myc, and combine them into a cocktail. Add into the cell mixture. Mix gently pipetting up and down (see Note 4).
5. Plate 1 mL of the cell/virus mixture onto a single well of a gelatin-coated 6-well plate, and incubate for 24 h.
6. After this time, wash with warm MEF medium, replenish with 2 mL of fresh MEF medium, and continue incubation.
7. Leave the culture until iPS colonies appear. Once they do (around day 7), pick colonies with a 200 μL pipet.

After post-viral transduction, the cells are grown on X-ray radiation inactivated mouse embryonic fibroblast (MEF) feeder layer with mTeSR1. After that, cells were adapted to grow onto Matrigel.

3.5.3 Hepatoblast-Like Cell Production

Differentiation of iPS cells into hepatoblast-like cells is induced as previously described [4].

1. Pig pluripotent stem cells should be maintained on Matrigel-coated 100 mm tissue culture dishes using mTeSR1 (see Note 5).
2. After 50% of confluence, wash the cells with sterile PBS−, and incubate the cells for 2 min with 3 mL of PBS−/0.02% EDTA at room temperature. As soon as the cells begin to detach, remove the PBS−/0.02% EDTA solution, and wash the plate with 6 mL of mTeSR1.
3. Pipet the cell solution to obtain small clusters, centrifuge cells at 200 × *g* for 5 min, and suspend in mTeSR1.

4. Transfer the cell suspension to a suitable number of wells of a 6-well matrigel-coated culture plate. Culture the cells overnight (see Note 6).
5. Change the culture medium to RPMI 1640 medium supplemented with 2% B27 (without insulin), 100 ng/mL Activin A, 10 ng/mL BMP4, and 20 ng/mL FGF2, and culture for 2 days with daily medium changes.
6. Substitute the culture medium again to RPMI 1640 with 2% B27 (without insulin), 100 ng/mL Activin A for 3 days with daily medium changes.
7. Culture the definitive endoderm cells with RPMI 1640 containing 2% B27 (with insulin), 20 ng/mL BMP4, and 10 ng/mL FGF-2 for 5 days with daily medium changes.
8. Culture the hepatic progenitor cells with RPMI 1640 containing 2% B27 (with insulin) and 20 ng/mL HGF for 5 days with daily medium changes.

By day 16 of the differentiation protocol, the cells should display a morphology that resembles hepatoblasts, with 80–90% of the cells expressing AFP and, quite commonly, some lipid droplets within the cytoplasm of the cells.

3.6 Scaffold Preparation and Sterilization for Bioreactor

1. After liver decellularization, the left and central lobes are removed, to decrease the size of the scaffold and, consequently, the total cells needed to recellularize it.
2. A 4-0 silk suture is passed through the vascular structure of the left lobe to ligate it. The vessels are then cut, and the lobe is removed. This same procedure is performed to remove the central lobe.
3. The diaphragm should be trimmed as much as possible.
4. The remnant right lobe scaffold is then put in a 150 cm culture dish with 20 mL of distilled water.
5. The scaffold is then sterilized with X-ray at 160 kV, 6.3 mA, for 99.9 min (see Note 7).

3.7 Bioreactor Assembly and Recellularization

For liver recellularization, it is necessary to assemble the setup described in Fig. 3. The process consists of three phases: the first is the priming, the second is cell seeding, and the third is the maintenance of the recellularized scaffold (for 7 days).

1. All the components used for the bioreactor setup are sterilized (see Note 8).
2. All the components are assembled in a culture biosafety cabinet, using sterile gloves, sterile lab coat, and a mask, to reduce contamination.

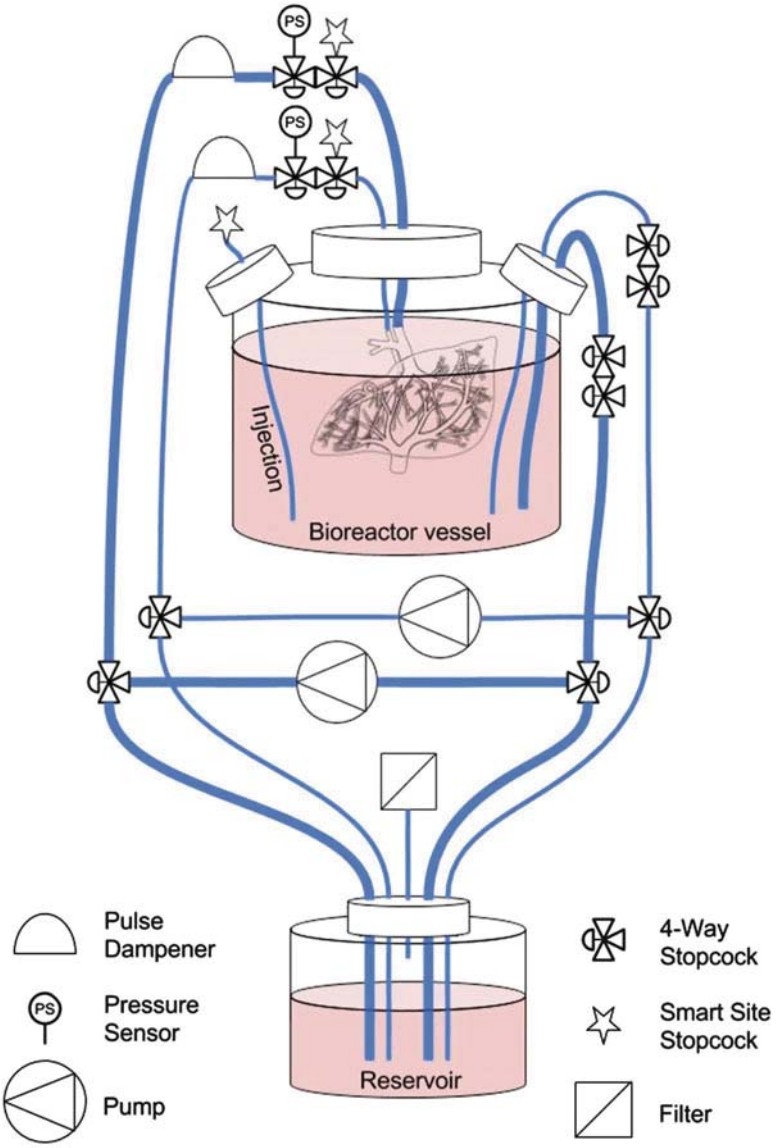

Fig. 3 Schematic diagram of the bioreactor system. Cells are injected through the injection tube to the media within the spinner flask. Then, they are perfused by the peristaltic pumps all across the circuit to the liver, entering the organ through the portal vein and the hepatic artery

3. The tubing is connected to the four-way stopcocks and smart site stopcocks using male and female Luer locks. The pulse dampeners are linked to the tubing.
4. Once the tubing is connected to the bioreactor vessel and the reservoir and the circuit is closed, it is primed with culture medium using a 20 mL Luer-lock syringe, to remove the air from the system.

5. The liver is attached to the tubing through the portal fitting and the hepatic cannula and is suspended in the culture medium within the spinner flask.
6. The bioreactor system is transferred to an incubator at 37 °C/5% CO_2 and attached to the peristaltic pumps.
7. The liver is then perfused at low-pressure conditions overnight before the cell seeding: 25 mmHg in both lines.

3.8 pUVEC Preparation for Bioreactor Seeding

1. Aspirate the EGM-2 media from the 150 cm culture dish, and wash with 10 mL of PBS without calcium and magnesium.
2. Add 7 mL of trypsin/EDTA and incubate for 4 min at 37 °C.
3. Collect the trypsin with the detached cells, and transfer to a 50 mL conical tube.
4. Rinse the dish with 7 mL of DMEM +10% FBS + 1% P/S for any leftover cells, and transfer to the 50 mL conical tube.
5. Centrifuge at 300 × *g* for 5 min. Aspirate supernatant and reconstitute the pellet with 10 mL of bioreactor media.
6. Cell counting.
7. Keep the cells on ice until ready to be injected in the bioreactor system.

3.9 pMSC Preparation for Bioreactor Seeding

1. Aspirate the DMEM/F12 media from the 150 cm culture dish, and wash with 10 mL of PBS without calcium and magnesium.
2. Add 7 mL of trypsin/EDTA and incubate for 4 min at 37 °C.
3. Collect the trypsin with the detached cells and transfer to a 50 mL conical tube.
4. Rinse the dish with 7 mL of DMEM with 10% FBS and 1% P/S for any leftover cells, and transfer to the 50 mL conical tube.
5. Centrifuge at 300 × *g* for 5 min. Aspirate supernatant and reconstitute the pellet with 10 mL of bioreactor media.
6. Cell counting.
7. Keep the cells on ice until ready to be injected in the bioreactor system.

3.10 iPS-Derived Hepatoblast Preparation for Bioreactor Seeding

1. Aspirate the media.
2. Add 3 mL of TrypLE for 10 min at room temperature.
3. Collect the TrypLE with the detached cells, and transfer to a 50 mL of conical tube.
4. Inactivate with DMEM/F12 with 10% FBS and 1% P/S.
5. Centrifuge 200 × *g* at room temperature, and reconstitute the pellet with 10 mL of bioreactor media.
6. Cell counting.

7. Keep the cells on ice until ready to be injected in the bioreactor system.

Once all cell types are detached and counted, they should be centrifuged again. Pellets should be mixed with 10 mL of bioreactor media for scaffold seeding and supernatants discarded.

3.11 Scaffold Seeding

1. An ammount of approximately 60 million hepatoblasts, 45 million pUVECs and 12 million pMSCs (ratio 10:7:2) are co-seeded through the portal vein and the hepatic artery of the piglet liver scaffold by perfusion at 60 mmHg in the hepatic artery and 25 mmHg in the portal vein.
2. All types of cells used are put together in 10 mL of bioreactor media (see Note 9) and injected into the bioreactor through the injection tube. Peristaltic pumps will recirculate the cells to the portal vein and hepatic artery.
3. Cells are seeded in two steps, separated by 4 h.
4. After the second seeding, a maintenance pressure is set up for 1 week, to maintain a specific fluid flow inside the scaffold that allows the attached cells not to detach. A set point of 25 mmHg will be used for both lines.
5. The scaffold is left in the bioreactor system for 1 week, changing media every 2 days.
6. After 1 week, the reseeded scaffold needs to be perfused with 4% paraformaldehyde for 15 min, after which small pieces are taken for tissue processing.

4 Notes

1. Porcine infra-hepatic vena cava penetrates the liver right lobe. Some care is needed not to disrupt this hepatic lobe.
2. Depending on each liver, it might be necessary to prepare more or less detergent.
3. Colonies usually appear between 5 and 21 days after pUVECs isolation.
4. A four-in-one mono-cassette virus is recommended. 1 μg/mL of doxycycline should be added in every medium change to induce gene expression. The amount of virus added is calculated using a multiplicity of infection (MOI) of at least 20. To determine the desired amount of virus: virus volume (μL) required = [(# pMSCs seeded for infection)/virus titer (U/mL)] × [(desired MOI)/1 mL] × 1000 μL.
5. It is essential to start the differentiation protocol of iPCs cells into hepatoblast-like cells without minimal morphological evidence of differentiation.

6. The cell density at the initiation of differentiation usually has a dramatic effect on differentiation efficiency, so the best density should be determined empirically.
7. It is desirable to use gamma irradiation for scaffold sterilization, but this procedure is not always available.
8. Tubing, spinner flask, and pulse dampener are sterilized by steam at 121 °C. Some other components cannot be autoclaved: four-way stopcocks and smart connectors are sterilized with ethylene oxide; the pressure sensor is cleaned with a bactericide solution for 15 min and then washed with sterile PBS. The outer part is cleaned with ethanol.
9. Before loading the cell suspension into a Luer-lock syringe, it is vital to resuspend the cells carefully to reduce cell aggregation which could obstruct the scaffold vascular network.
   In this chapter, we describe a seeding method in which cells are seeded with continuous perfusion. Some other authors have used multistep infusion systems, where cells are delivered directly inside the scaffold.

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