PREPARATION OF PRIMARY CULTURES OF ADULT AND JUVENILE CATFISH (*ICTALURUS PUNCTATUS*) HEPATOCYTES

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SUMMARY: We describe a modified perfusion and collagenase dissociation in a trypsinizing flask for hepatocyte isolation from adult or juvenile catfish liver. When cells isolated in this manner are allowed to attach to plastic culture dishes in serum-free medium, good viabilities and prolonged function can be obtained.

Key words: juvenile catfish; primary liver cell cultures; parenchymal hepatocytes; serum-free media; adult catfish.

I. INTRODUCTION

The use of fish hepatocytes in metabolic and toxicologic studies has been restricted primarily to freshly isolated adult cells kept in suspension cultures (3-5,7,9). The use of primary monolayer cultures has been hampered as cells do not readily attach to plastic culture dishes or other substrata typically used with mammalian systems (6). Little work has been done on development of primary monolayer culture systems for juvenile fish cells; although sexually immature salmon hepatocytes have been studied in primary monolayer cultures (8). For toxicologic or carcinogenicity testing applications, an in vitro system using juvenile cells will be important as toxic responses are age specific, with juveniles usually being more sensitive than adults. The usual perfusion method employed involves cannulation and a continuous retroperfusion using collagenase as the dissociative enzyme (2). This method is difficult to adapt to juvenile fish because of their small size.

Here we report an isolation method that has been modified from a procedure used for postnatal rats (1). In the catfish it is efficient, less costly than the continuous perfusion, consistently repeatable, easily adaptable to both adult and juvenile channel catfish (*Ictalurus punctatus*) hepatocytes, and yields comparable cell numbers of quality as high as that of the cannulation procedure. When utilized with the modifications specified for our serum-free medium, good viability, prolonged survival with differentiated function, and cell attachment to plastic culture dishes occurs.

II. MATERIALS

A. Chemicals and culture medium

1. Dissociation medium

   Hanks' balanced salt solution, no. 9230 Irvine^1^

2. Culture medium

   Dry powder minimum essential media in Hanks' salts, no. 9480^2^
   NaHCO₃, no. SX325 MCB, Matheson Coleman & Bell^3^
   1-glutamine solution, no. 9317^4^
   Penicillin-streptomycin solution, no. 9366^5^
   Amphotericin B (Fungizone), no. 9352^6^
   HEPES buffer, no. 96760^7^
   Sterile non-essential amino acids MEM-100x, no. 9304^8^

3. Miscellaneous

   Ethyl alcohol 95%
   Benzalkonium chloride, no. B-1383^9^
   Wescodyne, no. NJ131 AMSCO^10^

B. Glassware and plastics

   Sterivex-GS filter unit, 0.22 µm, no. SVGSB1010 Millipore^11^
   Tissue culture dishes, 35 X 10 mm, no. 25000 Corning^12^
   Culture tubes, 16 X 150 mm, no. T1290-8 Baxter^13^
   Plastic pipette, serological: 1 ml, no. 324-202; 5 ml, no. 257-008; 10 ml, no. 257-009 CMS^14^
   Pipette, transfer (Pasteur), 5½ in, no. 13-678-20A; 9 in, no. 13-678-20C Fisher^15^
   Trypsinizing flasks, 75 ml, no. 355213 Wheaton^16^ or 150 ml, no. 355214^17^; with a 22-mm star-shaped magnetic stirrer, no. 6600-0022 Nalge^18^
   Sterile disposable petri dish, 100 X 15 mm, no. 08-757-13^19^

C. Equipment

   Inverted compound microscope, Olympus^20^
   General lab bright field microscope^21^
   Clinical centrifuge, model HN-S IEC^22^
   Hot plate with stirrer, model PC351^23^
   Pipet-aid, no. 4-00-110 Drummond Scientific^24^
   CO₂ incubator, model 417 Lab-line^25^
   Tissue culture enclosure, no. 11000 Labconco^26^
Laminar flow hood, Contamination Control

D. Miscellaneous

Hemacytometer, Bright-Line, Reichert-Jung
Filter holder, syringe type, 25 mm, with stainless steel mesh screen, no. 4320 Gelman
Syringe, sterile disposable: 10 cc, no. 7340; 30 cc, no. 7450A Pharmaseal
Hypodermic needle: 27G1/4, no. 8881-200508
Monoject; 26G1/2, no. 5111 Becton Dickinson
Scissors: surgical, 4 1/2 in. straight, no. 08-9409;
operating, 6 1/2 in. straight-blade, no. 13-810-029
Forceps, stainless steel dressing, 6 in., no. 13-812-409
Dog comb, fine toothed stainless steel, 6 in., no. LK77110 fine Lambert-Kay
Rubber mallet, head 2 X 3 1/2 in.
Catfish, Ictalurus punctatus, Tank Hollow Fisheries

III. PROCEDURE

A. Preparation of media

1. Dissociation medium, Hanks’ calcium-free balanced salt solution (HBSS) with bovine albumin and collagenase
   a. Dilute 10 ml Irvine sterile 10x HBSS stored at 4°C in 90 ml sterile double-distilled water.
   b. Add 0.5 g bovine albumin fraction V and 0.05 g (for adults) or 0.03 g (for juveniles) collagenase.
   c. Let sit until chemicals sink to the bottom of the flask, then swirl gently to mix.

2. Culture medium, minimum essential medium (MEM) in Hanks’ salts
   a. Dissolve MEM dry powder in 1 liter of double-distilled water.
   b. Add 0.35 g NaHCO3, 10 ml sterile non-essential MEM-100x amino acids, and 1.19 g HEPES buffer.
   c. Sterilize the MEM by filtration under positive pressure through the Sterivex-GS filter unit.
   d. Store in 100-ml bottles at 4°C.
   e. As needed, add to 100 ml of MEM 1 ml sterile Fungizone solution (250 µg/ml H2O), 10 ml sterile penicillin-streptomycin solution (10 000 U/ml/10 000 µg/ml), and 1 ml sterile L-glutamine solution (29.2 mg/ml) on the day of use.

3. Alcohol solution
   a. For approximately 1 liter add 700 ml 95% ethyl alcohol, 246 ml double-distilled water, and 7.5 ml of 0.13% benzalkonium chloride solution.

4. HCO3-rich HBSS
   a. Add 2 to 4 ml 1 N NaHCO3 to 10 ml sterile (1X) HBSS without albumin or collagenase.
   b. Place in a sterile vial with a 5 1/2 in. Pasteur pipette to dispense solution.

B. Isolation and dissociation of liver

1. For a typical culture, one adult (227 to 680 g) or 5 to 10 juvenile (10 to 36 g) channel catfish are required. Adults are paralyzed by a blow to the head with the mallet, juveniles are stunned by a blow to the snout then pitched with a dissecting needle by entering the cranial area between the eyes. Place fish ventral surface up, wash ventral surface with Wescodyne, and rinse with the alcohol solution.

2. Transfer fish to the sterile tissue culture enclosure (sterilize working surfaces with the alcohol solution). Using the scissors (large for adults or small for juveniles) make an incision midline at the caudal end, cut toward the head stopping just below the pectoral girdle. Make lateral cuts along the pectoral girdle and at the base of the incision on the caudal end so that the body wall may be pulled to either side.

3. Isolate the hepatic portal (left side for adults slightly more central for juveniles). Push the organs to one side for easy access to the portal vein.

4. Enter the portal vein with the 26-g (adults) or 27-g (juveniles) needle attached to the 30-cc (adults) or 10-cc (juveniles) hypodermic syringe. Perfuse the liver with 15 to 20 cc (adults) or 1 to 4 cc (juveniles) dissociation medium with 0.05% (adults) or 0.03% (juveniles) collagenase. The liver will blanch. Continue perfusion until complete blanching occurs. Clamp off the portal vein with the sterile forceps and hold to prevent blood from reentering the liver.

5. Cut supporting connective tissue with the small scissors to free the liver. Cut the portal vein last. Clean off as much of the nonhepatic tissue as possible.

6. Rinse the liver in HBSS without collagenase and bovine albumin to remove red blood cells. If using adults, transfer the liver to a sterile petri dish containing HBSS and tease the outer hepatobiliary capsule with the sterile stainless steel fine-tooth dog comb. If using juveniles omit this teasing step and place 10 ml of HBSS in a small vial, and dip.

7. Transfer the liver with sterile forceps to a trypsinizing flask containing 20 ml of dissociation medium with 0.05% (adults) or 0.03% (juveniles) collagenase.

8. At room temperature stir the tissue on a hot plate-stirrer. The setting should provide a gentle agitation of the tissue.

9. After 5 min of incubation, using a sterile 9-in. Pasteur pipette remove the supernatant and discard.

10. Add 20 ml of dissociation medium to the sterile trypsinizing flask and stir for 20 min, adding HCO3-rich HBSS as needed to maintain pH 7.6.

11. After the 20-min incubation, remove the suspended cells with a sterile 9-in. Pasteur pipette in the laminar flow hood. Go below the clear red layer to remove the supernatant. Place the supernatant in the sterile culture tubes and centrifuge for a 5-min 400×g spin. All the remaining steps, except for centrifugation, are done in the laminar flow hood using sterile equipment.

12. After the spin, remove the supernatant with a Pasteur pipette, being sure to wash down the sides