Title: A Simple Method for Rat Pancreatic Islets of Langerhans Isolation

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Abstract

The isolation of pancreatic islets of Langerhans from rodents is a fundamental technique in diabetic and related research fields. Since 1967, when Lacy and Kostianovsky introduced a new collagenase based method, the achievement of a high yield islet isolation method has become one of the most favored issues by scientists.

In fact, a reliable investigation depends on adequate and intact islets. With all respect to previous methods and protocols, here we introduce a simple method for isolation of rat pancreatic Islet of Langerhans that consists of three major steps: (1) collagenase perfusion into the pancreas via the common bile duct; (2) pancreas digestion, and (3) the purification of islets using ficoll gradients. This protocol provides an efficient and reproducible method to isolate intact islets with the least amount of contamination to exocrine tissues. Additionally, in comparison with previous methods, our protocol is simple, less intensive, time consuming, and low-cost. The entire procedure takes 30-45 minutes which is important for conserving islets viability. Additionally, this procedure can simply be applied by any individual who attempts to isolate rat the islets of Langerhans for the first time.

Keywords: Rat, Pancreas, The islets of Langerhans, Isolation**.**

A. Equipments

1. Rodent surgery board
2. 10 cc syringes
3. Sterile gloves
4. Face masks
5. Paper towels
6. Dissecting scissors/micro-dissecting scissors
7. Tissue forceps
8. Micro-mosquito forceps
9. Ultrafine forceps
10. Ultrafine straight scissors
11. Scalp vein needle (butterfly needle, if possible) or 27-30 gauge syringe
12. Surgical microscope
13. Bucket with ice
14. 37°C water bath
15. Scale (accurately measures in milligrams)
16. Petri dishes/counting dishes
17. 20 cc syringe
18. 50 ml conical tubes
19. 50 ml tube racks
20. P1000 pipette with standard precision tips
21. Disposable serological pipettes (1, 5, 10, 25 ml)
22. 0.22-µm pore size filter (Orange, cat. no. 1520012)

B. Materials

1. Hank's balanced salt solution (HBSS) (1X) (Invitrogen, cat. No.14185)
2. Fetal bovine serum (FBS, Hyclone, cat. no. SH30070.03)
3. Collagenase V (Sigma-Aldrich, cat. no. C9263)
4. RPMI 1046 medium (Invitrogen, cat. no.51800-035)
5. Penicillin/streptomycin (Invitrogen, cat. no. 15070-063)
6. L-glutamine (L-Gln, Invitrogen, cat. no. 25030-024)
7. Non-essential amino acids (NEAAs, Invitrogen, cat. no. 11140-035)
8. Lymphodex (Lymphocyte Separation Medium, Quest biomedical, 002041600)
9. 70% ethanol

Reagent Setup

A. Solution I: Collagenase solution

1. Dissolve 20 mg Collagenase V in 10 ml HBSS 1x to a final concentration of 2 mg/ml
2. Sterilize by filtration through a 0.2 µm filter
3. Maintain on ice until further processing

B. Solution II: Washing solution

1. Combine the following chemicals:

- 45 ml HBSS 1x
- 5 ml FBS
- 500 µl of penicillin/Streptomycin

2. Sterilize by filtration through a 0.2 µm syringe filter
3. Maintain on ice until further processing

C. Solution III: Culture media

1. Combine the following chemicals:

- 45 ml RPMI
- 5 ml FBS
- 500 µl penicillin/streptomycin

2. Sterilize by filtration through a 0.2 µm syringe filter

3. Keep on ice until further processing

D. Anesthetic solution

1. Mix 2 ml of ketamine and 1 ml of xylazine

The dosage of ketamine/xylazine used to induce anesthesia is typically 0.005 ml/g body weight. Euthanasia is induced by doubling this volume.

Typically 0.005 ml/g of body weight is used as a measure for the dose of ketamine/xylazine to anesthetize the animal.

Procedures

A. Surgical procedure

1. Anesthetize animal by IP injection of anesthetizing solution (ketamine/xylazine).

2. Ensure that the animal is properly anesthetized by toe pinch.

3. Lay the animal in appropriate position so the surgeon can visualize the abdomen.

4. Spray 70% ethanol on the animal to reduce the chance of contamination during surgery. It is better to immobilize the animal.

5. Open the abdomen in a V shape, starting from the lower abdomen in two directions and extend scissions to the lateral portions of the diaphragm in order to expose all organs in the peritoneal cavity.

6. Displace the internal organs, such as intestine and stomach, to the right side.

7. Secure the liver with gauze or swab.

8. Find the common bile duct (CBD), which is extended from the liver to the intestine.

9. Locate where the CBD enters the small intestine.

10. Using a Bulldog clamp, clamp the CBD near its junction with the small intestine. Be careful not to occlude the pancreatic duct. Clamping the duodenum on either side of the junction with the CBD is another alternative.

11. Fill a 5 ml syringe with solution I (enzyme solution).

12. Make a scission in the CBD at the most superior portion of the CBD.

13. Inject 4-5 ml of solution I into the CBD for full inflation of the pancreas. Full inflation of the pancreas in this step greatly influences the final yield. Remove the pancreas and place it into a 50 ml conical tube. To remove the pancreas, begin at the point where the CBD is attached to the duodenum. Then, cut the CBD from its attachment to liver. Continue removing the pancreas from the stomach until the spleen is reached. It is better not to remove the pancreas from spleen at first since the spleen can be used as a handle. Remove the spleen when the tissue (pancreas) is to be placed into the conical tube that contains 5 ml of solution I (Collagenase solution).

14. Keep the conical tube that contains tissues on ice until the digestion procedure.

B. Digestion and purification procedure

1. Incubate tissues for 20 minutes at 37°C in a water bath. Incubation time varies among different lots of enzymes, but is usually 15–20 minutes.

2. Every 5 minutes remove the tube, shake firmly 2-4 times, and then return the tube to the water bath.

3. After the incubation time is completed, fill the tube with 10 ml of solution II to dilute Collagenase and stop the digestive process.

4. Shake the tube vigorously for 30 seconds to mechanically digest the rest of the exocrine tissues and to complete tissue separation.

5. After mechanical digestion, the mixture should be homogenous and resemble to the pea soup. Fat tissue is also isolated with the pancreas and will remain undigested; therefore it is normal to see undigested pieces of fat in the final soup.

6. Pour the soup through the sieve. In this step, undigested tissues cannot pass through the strainer therefor digested tissue can be collected.

7. Wash the surface of strainer with solution II to wash all the remaining islets.

8. Immediately transfer the filtered solution into a 50 conical tube.

9. Centrifuge for 1 minute at 1000 rpm.

10. Discard the supernatant.

11. Resuspend the pellet in 10 ml solution II.

12. Centrifuge for 1 minute at 1000 rpm.

13. Repeat steps 11-12 for two more times.

14. Resuspend the pellet in 20 ml ficoll (lymphodex) solution. Resuspend the tissue completely by proper pipetting and make sure that there are the least tissue clamps after pipetting. Tissue should become almost homogenous with the ficoll. If not, attached exocrine tissues to islets affect the islets' density and make them to stand in wrong gradient layer.

15. Fill the 20 ml syringe with solution III.

16. Turn the tube 45 degree and add solution III slowly via a syringe. Avoid mixing the medium and ficoll with each other. As a result two separate phases will form, the upper one is ficoll and the lower one is medium
17. Centrifuge at 2000 rpm for 10 minutes with no acceleration and breaking speed.
18. After the centrifuge is completed, the isolated islets stand in the intermediate phase between the ficoll and the medium. Due to toxicity of ficoll for cells this step should performed quickly to avoid cell apoptosis. Transfer the collected islets to another 50 ml conical tube.
19. Resuspend the collected islets in 10 ml of solution III. It is helpful to separate the collected fine exocrine tissue, if any, also this procedure helps diluting the pulled up ficoll.
20. Centrifuge for 1 minute at 1000 rpm.
21. Decant the supernatant and replace it with 10 ml of solution III.
22. Repeat steps 19-21 twice more.
23. Transfer the islets to a sterile petri dish under a culture hood to incubate or pick up the islets under a dissecting or light microscope. Use a suspension culture dish so the islets do not stick as they would in treated tissue cultures.

Troubleshooting advice can be found in Table 1.

ANTICIPATED RESULTS

To evaluate morphology of isolated Islets of Langerhans and expression of markers, we analyzed the islets for markers. We believe that protocol presented here offers a opportunity to acquire reasonable number of islets with very good purity, in addition to less time for each rat, 30-45 min. (Fig. 4).

Figure legends

Figure 1. The isolated islets of Langerhans. The morphology of freshly isolated islets (A and B)

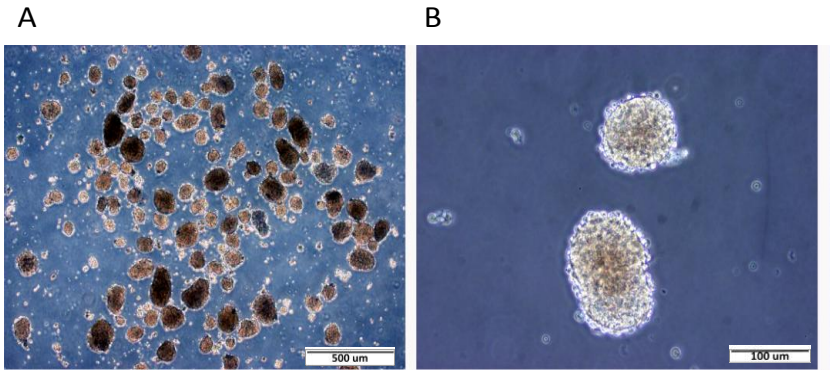
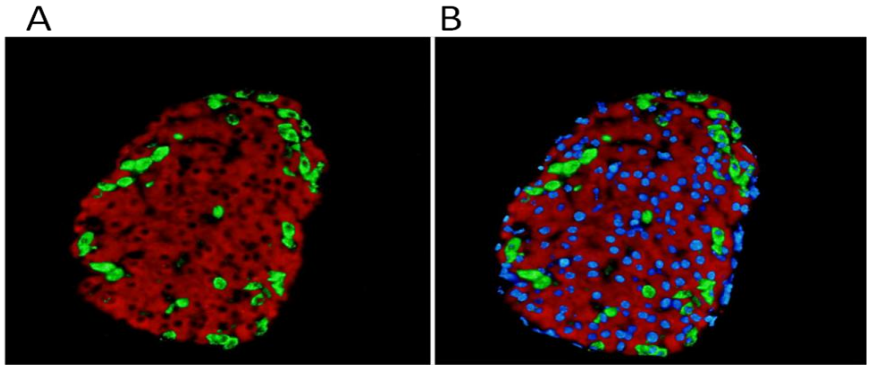


Figure 2. The characterization of isolated islets. (A) Immunofluorescence staining for expression of Insulin (red) and Glucagon (green). (B) Nuclei were stained with DAPI (blue). All magnifications: ×20



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