Chapter 23

Formulation of Selected Renal Cells for Implantation into a Kidney

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Abstract

Delivery of cells to organs has primarily relied on formulating the cells in a nonviscous liquid carrier. We have developed a methodology to isolate selected renal cells (SRC) that have provided functional stability to damaged kidneys in preclinical models (Kelley et al. Poster presentation at 71st scientific sessions of American diabetes association, 2011; Kelley et al. Oral presentation given at Tissue Engineering and Regenerative Medicine International Society (TERMIS)—North America annual conference, 2010; Presnell et al. Tissue Eng Part C Methods 17:261–273, 2011; Kelley et al. Am J Physiol Renal Physiol 299:F1026–F1039, 2010). In order to facilitate SRC injection into the kidney of patients who have chronic kidney disease, we have developed a strategy to immobilize the cells in a hydrogel matrix. This hydrogel (gelatin) supports cells by maintaining them in a three-dimensional state during storage and shipment (both at cold temperatures) while facilitating the delivery of cells by liquefying when engrafting into the kidney. This chapter will define a method for the formulation of the kidney epithelial cells within a hydrogel.

Key words Kidney disease, Renal epithelial cells, Gelatin, Hydrogel

1 Introduction

There are several primary diseases that impact the function of the kidney. As these diseases progress, the kidney goes through several stages of tissue damage (stages I–V). For later stages of kidney damage, the treatment modalities are limited to either dialysis or eventually, kidney transplant. Based on data generated in animal models (1–4), the transplantation of autologous, homologous selected renal cells (SRC) into the kidney at an early stage of the degeneration of the kidney (stage III or early stage IV) should prolong kidney health. Hence, the transplantation of these cells has a great potential in delaying the eventual use of dialysis by off-setting the loss of function of the kidneys.

Cell transplantation has been used for the amelioration or treatment of many diseases or tissue injury. Some of the

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applications include the implantation of chondrocytes for cartilage repair (5), delivery of non-encapsulated pancreatic islets for the treatment of Type I diabetes (6), and the injection of mesenchymal stem cells for the repair of heart muscle tissue (7). All of these therapies have relied on delivering the cells in a nonviscous solution (such as saline or cell culture media). Some of the challenges with this approach of cell delivery have included the potential for cell settling during the injection phase (which can reduce the even distribution of the cells upon implant) and short-term shelf life due to product settling and cell aggregation.

Gelatin is a non-cross-linked collagen that has been used for many different medical applications. The biocompatible nature of gelatin has been extensively reviewed and this material has been used for in vivo applications (8). One can control the gelation properties of the gelatin (formation of a hydrogel) depending on the concentration and bloom (a measure of force (weight) required to depress a standard plunger of 12.5 mm diameter into the surface of the gel at a distance of 4 mm (AOAC standard)) of the gelatin as well as its temperature. Previously (9–12) we have shown that gelatin is biocompatible with SRC both in vitro and in vivo.

This chapter will describe a method to suspend the cells in gelatin mixed in a phosphate buffered saline solution for storage and shipping at a cold temperature. This formulation has the unique property of remaining in a gelatinous state at a cold temperature and upon warming up to room temperature begins to liquefy (17–21°C). Hence, the product can be shipped and stored cold keeping the cells in a suspension. When required, the product can be removed from the cold and warmed to room temperature where it begins to liquefy.

2 Materials

2.1 Formulation of Rat Renal Cells

2.2 Cell Culture Media and Reagents

1. Dulbecco's Modified Eagle Medium, high glucose (DMEM-HG), containing sodium pyruvate.
2. Keratinocyte Serum-Free Medium (KSFM) containing L-glutamine and supplied with prequalified human recombinant epidermal growth factor 1-53 (EGF 1-53) and bovine pituitary extract (BPE) in separate packaging (Invitrogen) (see Note 1).
3. Fetal bovine serum (FBS).
4. Insulin–Transferrin–Selenium G solution (ITS) 100× (10 mL per 1 L Media).

5. Antibiotic/Antimycotic, 100× (Invitrogen) (10 mL per 1 L Media).
6. Renal Cell Culture Media—50:50 Mix of DMEM and KSFM with 5% FBS, with EGF and BPE, and ITS.
7. 0.25% Trypsin–EDTA.

2.3 Tissue Digestion

1. Collagenase Type IV—300 U in 600 μL.
2. Dispase, 5 mg/mL concentration, in Hank's buffered saline solution.
3. Neutralizing Media (Renal Cell Culture Media).

2.4 Cell Separation

1. Iodixanol (Optiprep™ 60% wt/vol) in KSFM (see Notes 2 and 3).
2. Dulbecco's phosphate buffered saline (DPBS) (For washing of cells after gradient separation).
3. Steriflip (Millipore).

2.5 Cell Formulation

1. Porcine Skin Gelatin (Type A) from Gelita USA (Sergeant Bluff, IA, USA).
2. Mix Gelatin with DPBS from 0.625 to 1.5% (see Note 4).

2.6 Creating a Gel

1. Syringe (1–10 cc syringes).
2. Sterile luer lock cap.

2.7 Equipment

1. Class 100 inverted laminar flow biological safety cabinet (BSC).
2. Tube rotator (Customized with an RPM setting of 2–25 RPM) (see Note 5).
3. Refrigerator with power outlet.
4. Humidified 37°C 5% CO_2 incubator.
5. Humidified 37°C 2% O_2 incubator.
6. Water bath (37°C).
7. Dry bath (28°C).
8. Swinging bucket centrifuge system (Sorvall, see Note 6).
9. Hot stir plate (needs 50°C).

2.8 Plastic Supplies and Cultureware

1. T-75, T-500 flasks.
2. 150 mm plastic petri dishes (pre-weighed).
3. Microcentrifuge tubes.
4. 15 mL centrifuge tubes.
5. Pipettes (1, 5, 10, 25 mL).
6. Syringes (1, 5, 10 mL).

7. Pipetters (Rainin or other brand; P10, P100, P1000).
8. Pipette tips for pipetters (see Note 7).
9. Glass beaker (100, 500 mL).
10. Magnetic stir bar.
11. 0.22 μm sterile filter units (Corning).

2.9 Stainless Steel Instruments and Surgical Material

1. Scissors.
2. Scalpel.
3. Forceps.
4. Hemostats.
5. Sterile gauze pads, 12-ply, 4×4 in.

3 Method

3.1 Rat Renal Cell Isolation Method

1. Rat kidneys are harvested after euthanasia from an appropriately certified vendor following all institutional guidelines for safe handling of animals.
2. Upon kidney arrival to the laboratory, aspirate the shipping medium and pour kidneys into a 150 mm dish.
3. Remove and discard connective tissue, calyx, and capsule (if applicable) around the kidney using forceps and scalpel.
4. Wash the kidneys one time in HBSS or PBS to remove any debris.
5. Manually mince together remaining kidneys using scalpel blade, making finely chopped slurry of tissue.
6. Add 1.0 g (±0.1 g) minced kidney tissue to each pre-weighed 50 mL conical tube.
7. Add 20 mL of pre-made digestion buffer to each 50 mL tube.
8. Perform two sequential digestions of the kidney tissue.
9. *Digestion 1:* Incubate digestion tubes at 37°C on a rocker for *20 min*.
10. Remove tubes from incubator/rocker and place in the BSC.
11. Allow undigested tissue to settle to the bottom of the tube via gravity.
12. Aspirate supernatant from each tube and discard.
13. Add 20 mL of warm digestion buffer (warmed to 37°C) to each 50 mL tube.
14. *Digestion 2:* Incubate digestion tubes at 37°C on a rocker for *30 min*.

15. Remove tubes from rocker/incubator, combine two digestion tubes into one, and run the contents of each tube through a 100 μm SteriFlip.
16. Transfer the cell suspension into a sterile bottle.
17. Neutralize the enzyme with an equal amount of neutralization buffer.
18. Centrifuge in 50 mL tubes at $300 \times g$ for 5 min (or in 225 mL tubes at $300 \times g$ for 8 min). Aspirate supernatant and discard.
19. Resuspend the cell pellet in desired amount of KSFM (break up clumps).
20. Count cells using a hemocytometer.
21. To clean up the tissue prior to plating the cells, the digested tissue is placed onto a 15% Mixing Gradient (OptiPrep).
22. Based on total remaining cell number, calculate how many 15 mL gradient tubes are needed to load 75×10^6 cells/tube (or in 50 mL tubes at 225×10^6 cells per tube).
23. Aliquot 75×10^6 cells into each 15 mL tube and bring each cell suspension up to 5 mL with KSFM or aliquot 225×10^6 cells into each designated 50 mL tube and bring each cell suspension up to 22.5 mL with KSFM.
24. Add an equal volume of 30% Optiprep to each tube in order to achieve a 15% OptiPrep solution.
25. Mix tubes by inversion six times.
26. Carefully layer 1 mL of PBS on top of Optiprep/cell mixture (or 5 mL for a 50 mL tube).
27. Centrifuge at $800 \times g$ for 15 min with NO brake.
28. Carefully remove the tube(s) and place back into the BSC.
29. Collect and combine all cell bands via a sterile transfer pipette into a clean 50 mL tube.
30. Aspirate and discard the remaining supernatant (leaving the cell pellet(s) in the tube(s)).
31. Resuspend all pellets with KSFM and combine with the bands collected above.
32. Add enough KSFM to reach a 4:1 ratio of KSFM to cell suspension (Split sample into multiple 50 mL tubes if necessary).
33. Mix tubes by inversion six times (see Note 8).
34. Centrifuge at $300 \times g$ for 5 min.
35. Aspirate supernatant leaving pellet in the tube.
36. Resuspend cell pellet with KSFM to desired volume. This is the 15% Band + Pellet sample.
37. Count cells using a hemocytometer.

38. The Band + Pellet cells are now ready to freeze, plate, analyze, or use for experimentation.
39. Plate 25,000 cells/cm^2 in a desired TC-treated vessel using complete Renal Cell Culture Media at a volume that is recommended by vessel manufacturer. (For example: T500 Nunc flask, 12.5×10^6 cells/flask, 100 mL of Renal Cell Culture Media.) The culture vessels containing cells (passage 0) are placed in a 5% CO_2 incubator at 37°C in a humidified environment (see Note 9). After 48 h, a complete media exchange occurs.

3.2 Harvesting the Cells for Formulation

1. After culturing the cells for 2–3 days, the cells are placed in a hypoxic (2% oxygen) environment overnight (see Note 10).
2. Prepare four gradient solutions (16, 13, 11, and 7%) of Iodixanol (Optiprep™ 60% wt/vol) in KSFM.
3. Each density step gradient requires a cell number ranging from 60 to 75 million cells per tube. Once the amount of cells has been determined, calculate the number of gradient tubes to be generated.
4. Make a density step gradient(s) by first pipetting 2 mL of 16% density gradient medium into a 15 mL conical centrifuge tube(s).
5. Carefully layer 2 mL of the 13% density gradient medium onto the 16% density gradient medium by tilting the tube at a 45° angle and letting the medium slowly run down the side of the tube. This will minimize mixing at the interface between the two different densities.
6. Once the 13% density gradient medium has been layered, continue with the 11% density gradient medium using the same layering method and finish with the 7% density gradient medium.
7. Once the gradients are formed, take care in moving as not to disturb the boundary interfaces.
8. Carefully pipette 2 mL of cell suspension containing between 60 and 75 million cells in KSFM medium on top of the step gradient. Continue until all of the gradients have been loaded with cell suspension.
9. Once the cells have been loaded, carefully place tubes into the centrifuge and spin at $800 \times g$ for 20 min without brake.
10. After centrifugation, collect tubes and visually inspect gradient bands to verify banding pattern.
11. Collect gradient bands by aspirating each band using either sterile bulb or 5 mL pipette.
12. Combine bands 2, 3, and 4 (B2, B3, and B4).
13. Wash the cells (B2, B3, and B4) three times using DPBS by centrifugation.

3.3 Formulation in Gelatin Hydrogel

1. Batches of gelatin are pre-made in DPBS at concentrations ranging from 0.625 to 1.5% v/v. Aliquot the gelatin into smaller vials to be used for the final formulation steps.
2. Briefly, gelatin is measured and dissolved in DPBS. The gelatin solution is dissolved at 50°C while being mixed and then sterile filtered using a 0.22 μm filter into a sterile container. The sterile gelatin solution is then aseptically added to smaller vials for storage at 4°C until use.
3. After washing, the pelleted SRC are re-suspended and counted to determine cell concentration to be formulated in the gelatin solution. At this time, heat up the pre-made gelatin solution aliquot(s) using a dry bath set at 25–28°C for >1 h.

3.3.1 After Counting the Cells a Final Wash Should Be Performed Using Gelatin

1. Following the final centrifugation, the gelatin solution supernatant is removed and sufficient volume of 0.625–1.5% gelatin is added to targeted volume/cell concentration with a maximum cell to gelatin ratio of 50:50. For example, an estimated packed volume for rat SRC is 300 million cells for a volume of 1.0 mL. If the total cell number required per injection is 20 million cells, then the minimum volume of product that could be injected into a kidney is 0.13 mL (0.067 mL of packed cells + 0.067 mL gelatin). Bring up the total volume to 0.13 mL by adding gelatin (see Note 10).
2. The final SRC product is used as a gel. To create the gel, add the gelatin/cell solution, use a small pipette tip that fits inside the luer lock of a syringe (see Note 11).
3. Carefully place a sterile luer lock cap onto the syringe. Make sure the cap is tight.
4. Place the container on a rotator at a minimum of 2.0 RPM in a 2–5°C environment overnight. The rotation maintains the cells in suspension during the gelation period.
5. Once gelation has occurred, the SRC/gelatin product can be removed from the rotator and stored cold until use (see Note 12).

4 Notes

1. KSFM comes with a bullet kit that is kept frozen until use. It is recommended to use the media within a couple of weeks after mixing the components.
2. Make sure Optiprep is made in an osmotically correct buffer such as KSFM.
3. Make solutions up prior to use.

4. The higher the concentration of gelatin, the stiffer the gel. At the higher concentration, the gelatin will take longer to liquefy at room temperature.
5. We used a modified electric drill with a polycarbonate disk attached to it.
6. Swinging bucket is preferable for establishing the different bands of the density gradient.
7. Small tips are used for loading the syringes with the cell/gelatin mixture.
8. If too little KSFM is added and/or if tubes are not mixed well, the Optiprep mixture will re-band.
9. Isolated rat renal cells do not passage (re-plate onto plastic). Hence, cell harvesting takes place at the end of the initial plating process (4 days after seeding onto the plastic).
10. The cells should not be greater than 90% confluent when placed in the low oxygen environment.
11. The user should take into account the dead volume of a syringe and the needle. For example, a 1 cc syringe with a 27 G needle has approximately 80 μL of dead volume. Hence, an additional 80 μL of product should be made to take this into account.
12. Ensure gelatin is chilled at or below 4°C for proper gelation. Do not exceed below 0°C during the process. Ensure gelatin + cells remain below 8°C for proper storage and shipping. Ensure gelatin + cells warm up to room temperature prior to injection. This will support the delivery of the cells into the kidney parenchyma.

References

1. Kelley R, Spencer T, Werdin E, Bruce A, Ilagan R, Wallace S, Watts B, Choudhury S, Cox B, Guthrie K, Jayo M, Bertram T, Presnell S (2011) Intra-renal transplantation of bioactive renal cells preserves renal functions and extends survival in the ZSF1 model of progressive diabetic nephropathy. Poster presentation at 71st scientific sessions of American diabetes association. www.tengion.com/news/documents/ADA%202011%20rwk_Tengion%20FINAL.pdf
2. Kelley RW, Werdin ES, Bruce AT, Wallace SM, Jayo MJ, Bertram TA, Presnell SC (2010) Bioactive renal cells augment renal function in the ZSF1 model of diabetic nephropathy. Oral presentation given at Tissue Engineering and Regenerative Medicine International Society (TERMIS)—North America annual conference. www.tengion.com/pdfs/Kelley-2010-TERMIS-FINAL.pdf
3. Presnell SC, Bruce AT, Wallace SM, Choudhury S, Genheimer C, Cox B, Guthrie KI, Werdin ES, Tatsumi-Ficht P, Ilagan R, Kelley RW, Rivera EA, Ludlow JW, Wagner BJ, Jayo MJ, Bertram T (2011) Isolation, characterization, and expansion (ICE) methods for defined primary renal cell populations from rodent, canine, and human normal and diseased kidneys. Tissue Eng Part C Methods 17:261–273
4. Kelley R, Werdin ES, Bruce AT, Choudhury S, Wallace SM, Ilagan RM, Cox BR, Tatsumi-Ficht P, Rivera EA, Spencer T, Rapoport HS, Wagner BJ, Guthrie K, Jayo MJ, Bertram TA, Presnell SC (2010) A tubular cell-enriched subpopulation of primary renal cells improves survival and augments kidney function in a rodent model of chronic kidney disease. Am J Physiol Renal Physiol 299:F1026–F1039
5. Zaslav K, Cole B, Brewster R, DeBerardino TM, Farr J, Fowler P, Nissen C (2009) A prospective

study of autologous chondrocyte implantation in patients with failed prior treatment for articular cartilage defect of the knee: results of the Study of the Treatment of Articular Repair (STAR) clinical trial. Am J Sports Med 37: 42–55
6. Shapiro J, Lakey J, Ryan E, Korbutt G, Toth E, Warnock G, Kneteman N, Rajotte R (2000) Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. NEJM 343:230–238
7. Williams AR, Hare JM (2011) Mesenchymal stem cells: biology, pathophysiology, translational findings, and therapeutic implications for cardiac disease. Circ Res 109:923–940
8. Van Vlierberghe S, Dubruel P, Schacht E (2011) Biopolymer-based hydrogels as scaffolds for tissue engineering applications: a review. Biomacromolecules 12:1387–1408
9. Basu J, Genheimer CW, Rivera EA, Payne R, Mihalko K, Guthrie K, Bruce AT, Robbins N, McCoy D, Sangha N, Ilagan R, Knight T, Spencer T, Wagner BJ, Jayo MJ, Jain D, Ludlow JW, Halberstadt C (2011) Functional evaluation of primary renal cell/biomaterial Neo-Kidney Augment prototypes for renal tissue engineering. Cell Transplant 20:1771–1790
10. Payne R, Knight T, Basu J, Rivera E, Robbins N, McCoy D, Jayo M, Halberstadt C, Jain D (2011) Bioresponse of mammalian kidney to implantation of polymeric materials. Society for biomaterials annual meeting & exposition. www.tengion.com/pdfs/Bioresponse-of-Mammalian-Kidney-to-Implantation-of-Polymeric-Materials.pdf
11. Basu J, Payne R, Rivera E, Robbins N, McCoy D, Knight T, Jayo MJ, Jain D, Halberstadt CR (2010) In vivo evaluation of biomaterials in mammalian kidney. Tissue Engineering and Regenerative Medicine International Society (TERMIS)—North America annual conference. www.tengion.com/pdfs/Basu-2010-TERMIS-Biomaterials-poster-FINAL.pdf
12. Basu J, Rivera E, Genheimer CW, Robbins N, Payne R, McCoy D, Guthrie KI, Sangha N, Knight T, Jayo M, Ludlow JW, Jain D, Halberstadt CR (2010) Mammalian kidney nephrogenic response to primary renal cell/biomaterial-based Neo-Kidney Augment™ Prototype. Tissue Engineering and Regenerative Medicine International Society (TERMIS)—North America annual conference. www.tengion.com/pdfs/Basu-2010-cell-biomaterial-NKA-poster-FINAL.pdf