Video Article

Development of a Refined Protocol for Trans-scleral Subretinal Transplantation of Human Retinal Pigment Epithelial Cells into Rat Eyes

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Abstract

Degenerative retinal diseases such as age-related macular degeneration (AMD) are the leading cause of irreversible vision loss worldwide. AMD is characterized by the degeneration of retinal pigment epithelial (RPE) cells, which are a monolayer of cells functionally supporting and anatomically wrapping around the neural retina. Current pharmacological treatments for the non-neovascular AMD (dry AMD) only slow down the disease progression but cannot restore vision, necessitating studies aimed at identifying novel therapeutic strategies. Replacing the degenerative RPE cells with healthy cells holds promise to treat dry AMD in the future. Extensive preclinical studies of stem cell replacement therapies for AMD involve the transplantation of stem cell-derived RPE cells into the subretinal space of animal models, in which the subretinal injection technique is applied. The approach most frequently used in these preclinical animal studies is through the trans-scleral route, which is made difficult by the lack of direct visualization of the needle end and can often result in retinal damage. An alternative approach through the vitreous allows for direct observation of the needle end position, but it carries a high risk of surgical traumas as more eye tissues are disturbed. We have developed a less risky and reproducible modified trans-scleral injection method that uses defined needle angles and depths to successfully and consistently deliver RPE cells into the rat subretinal space and avoid excessive retinal damage. Cells delivered in this manner have been previously demonstrated to be efficacious in the Royal College of Surgeons (RCS) rat for at least 2 months. This technique can be used not only for cell transplantation but also for delivery of small molecules or gene therapies.

Video Link

The video component of this article can be found at https://www.jove.com/video/55220/

Introduction

The human retina located at the back of the eye functions as a light sensory tissue and plays a critical role in vision perception. Retinal cell dysfunction or cell death therefore causes vision problems or permanent blindness. Disorders involving degeneration or dysfunction of cells in different layers of the retina are known as degenerative retinal diseases, among which AMD is the most common type and the leading cause of irreversible blindness in the elderly in developed countries[1,2]. The pathological process of AMD is associated with "drusen" accumulation between the RPE layer and the underlying Bruch's membrane, which in turn impairs RPE support of photoreceptor physiology, leading to neural retinal atrophy and vision loss[3,4,5]. Thus far, there is no cure for advanced dry (non-neovascular) AMD. The emergence of stem cell therapy as a new paradigm in regenerative medicine brings the hope of replacing the dysfunctional or dead RPE cells with stem cell-derived healthy cells. Indeed, extensive preclinical studies of transplanting stem cells (*e.g.*, human embryonic stem cell)-derived RPE cells into RPE-degenerative animal models have been performed[6,7], some of which have progressed to clinical trials[8,9] (NCT01344993, ClinicalTrials.gov). Recently, an alternative source of stem cells resident in the human RPE layer, the human RPE stem cells (hRPESCs), was identified by our lab and is currently being used in preclinical studies of hRPESC derived-RPE cell (hRPESC-RPE) transplantation therapy for AMD[10,11,12,13].

The subretinal injection technique is applied in the preclinical studies mentioned above by multiple groups, including our group. There are two general approaches for subretinal injection in animals: trans-vitreal and trans-scleral. The trans-vitreal approach has the advantage of the surgeon being able to directly observe the needle end as it penetrates the anterior eye, crosses the entire vitreal cavity adjacent to the lens, and penetrates the retina at the back to the eye to reach the subretinal space[14,15,16]. However, it requires disrupting the retina in two locations (anterior and posterior), carries the risk of damaging the lens, and can result in backflow of cells into the vitreous when the needle is retracted. In contrast, the trans-sclera approach, in principle, avoids involvement of the retina and vitreous, and backflow exits the eye. In pigmented rodents, the surgeon can initially observe penetration of the sclera, but after passage into the pigmented choroid, the needle end is no longer visible. Without direct observation, breaching the retina is common and can result in retinal dissection and delivery of cells and/or blood into the vitreous. Moreover, because the eye surface is curved, it is very difficult to know which needle angles and depths are most effective for trans-scleral injections.

In this visualized article, we introduce a trans-scleral subretinal injection method informed by the use of post-surgical evaluations with Optical Coherence Tomography (OCT), which allows a detailed examination of the injection site. Our trans-scleral injection technique utilizes defined locations, angles, and depths for injection needles to produce very low surgical trauma and high reliability. Here, we specifically demonstrate the injection of hRPESC-RPE cells into the subretinal space of the RCS rat, a pre-clinical model of human AMD. With this injection method, we successfully and consistently delivered hRPESC-RPE cells into the subretinal space of RCS rat eyes with a very high success rate. Injection of cells was previously found to result in preservation of RCS photoreceptors at least 2 months after injection[13]. This procedure is performed under the dissecting microscope and is easy to learn. It requires two people (a surgeon and an assistant) to perform the injection and the average time of injection for each animal is less than 5 minutes. The defined angles and depths for injection needles make it possible for laboratories, where OCT is unavailable, to achieve successful subretinal injection. It allows for highly reproducible subretinal access and can be used not only for cell transplantation, but also for drug delivery and gene therapies.

Protocol

All procedures involving animals have been approved by the Institutional Animal Care and Use Committee (IACUC) at the State University of New York at Albany.

1. Pre-injection Preparation

1. **Preparation of a hRPESC-RPE cell suspension**
   NOTE: All the following steps are performed in sterile tissue culture hood and familiarity with basic sterile technique is required.
   1. Isolate primary hRPE cells from human donor eyes aged 50-90 years and culture cells in 24-well plates[12]. Cryopreserve the cells at passage 1, thaw as needed and culture passage 2 (P2) cells for 4-5 weeks (**Figure 1A**) for injection.
   2. Remove the culture medium[12] and gently rinse wells twice with 500 µL pre-warmed 1X Dulbecco's Phosphate Buffered Saline without Calcium & Magnesium (1x DPBS-CMF) by adding 1X DPBS-CMF into wells using a 1,000 µL pipette and removing it using a vacuum.
   3. Add 300 µL trypsin/DNAse to each well. Incubate hRPESC-RPE cells in trypsin/DNAse (4 kU DNase per 1 mL of 0.25% trypsin-EDTA) for 4 min at 37 °C to dissociate the cells.
   4. Check the cells under the microscope to see if they have rounded up. Continue to incubate the cells in trypsin/DNase for an additional 2 min if they have not rounded up yet.
   5. Once the cells are rounded up, use a 1,000 µL pipette to triturate the detached cells from the well and transfer the trypsin/DNase containing detached cells into a 15-mL conical tube with equal volume of pre-warmed culture medium, to inactivate the trypsin/DNase.
   6. Rinse wells with pre-warmed 1X DPBS-CMF by gently pipetting up and down, especially around the edges of the well; then add these cells to the previous conical tube.
   7. Centrifuge the conical tube at 286 x g for 5 min at 4 °C to pellet the cells.
   8. Remove the supernatant and resuspend the cells with 1 mL culture medium.
   9. Count the cells using a hemocytometer.
   10. Centrifuge at 286 x g for 5 min at 4 °C to pellet the cells.
   11. Remove the supernatant and resuspend cells in sterile Balanced Salt Solution (BSS) at 50,000 cells/µL (to deliver 50,000 cells in 1 µL volume during subretinal injection).
   12. Transfer the final cell suspension (CS) into a 1.7-mL microcentrifuge tube and keep in an ice-water mixture until injection use.

2. **Preparation of cell injector**
   1. Insert a sterile 33-gauge beveled needle into the injection syringe and screw tightly to assemble the injector.
   2. Flush the injector with 100% ethanol 5-6 times.
   3. Flush the injector with 70% ethanol 5-6 times.
   4. Flush the injector with BSS 5-6 times.
   5. Mark the injector needle with a sterile black marker pen at a position of 600 µm away from the tip of the needle under the dissecting microscope (**Figure 1B**).
   6. Place the injector on a micromanipulator for injection.

2. Subretinal Injection

1. **Surgical area and animal preparation**
   1. Weigh a 4-5 week old RCS rat (60-100 g) and anesthetize it using the isoflurane vapor delivery system.
      NOTE: To induce anesthesia keep the isoflurane flow rate at 5%. Confirm the depth of anesthesia by pressing the paws, and then reduce the flow rate to 2-3% for anesthesia maintenance during surgery.
   2. Place a sterile surgery drape, with a heating pad underneath, on the stage of the dissecting microscope to set up a sterile surgical area.
   3. Transfer the rat to the surgical area and place the rat in a nose cone connected to the isoflurane system to maintain anesthesia.
   4. Cover the rat's body with gauze. Pinch the rat's toe to confirm full anesthesia.

2. **Trans-scleral subretinal injection under the microscope**
   1. Apply a drop of eye lubricant on the rat's unoperated eye.
   2. Position the rat onto its right side with its left eye facing the ceiling for injection, its head towards the surgeon's right hand and its back towards the surgeon.
   3. Trim any whiskers that cover the eye with small scissors.
   4. Drip a small amount of eye wash from the temporal side of the left eye and collect the excess at the nasal side with a cotton applicator to rinse the eye.

5. Dilate the pupil with 1% tropicamide and 2.5% phenylephrine (freshly made from 10% phenylephrine by diluting it in sterile 0.9% saline on the day of surgery) for a post-injection OCT exam by applying one drop of each.
6. Gently pull the skin surrounding the eye 4-6 times to open the eyelid so that the eye is slightly proptosed for easier access to regions posterior to the limbus.
7. Apply a drop of eye wash and keep the eye moist.
8. Gently triturate the CS (prepared in step 1.1.12) and load the injector with 1.2 µL CS. The extra 0.2 µL is used to compensate for injection backflow.
   NOTE: Based on our measurements, about 5,000-8,000 cells are lost in the backflow with an injection of 50,000 cells/µL that equals to about 10-16% of cell loss and an extra of 0.2 µL CS was injected to compensate this cell loss.
9. Place the injector filled with CS on a micromanipulator (or have an assistant hold it) vertically as RPE cells tend to sink easily in suspension.
10. Apply a drop of 0.5% proparacaine (local topical anesthetic) on the eye and remove the excess with a cotton applicator.
    NOTE: This step should suppress the corneal reflex and prevent the eye from blinking during subsequent steps.
11. Use forceps to grip the conjunctiva posterior to the limbus, rotate the eye nasally, and lift the conjunctiva to make a "tent".
12. Use scissors to cut the top of the "tent" off to make a small opening in the conjunctiva and expose the underlying sclera.
13. Use forceps to grip the edge of the remaining conjunctiva margin next to the limbus and rotate the eye nasally so that the pupillary axis is at an angle of about 30 degree relative to the table top (**Figure 1C**). Continued gripping of the conjunctiva margin is needed to provide a counter-force during needle insertions and to maintain the eye at an optimal angle.
14. To make a pilot hole for cell injection, position the end of a sterile beveled 31-gauge insulin needle at 1,200-1,500 µm posterior to the limbus with the opening of the tip facing up.
15. Adjust the angle of the insulin needle so that it is 10-15 degrees above the sclera (tangential to an imaginary plane at the intended injection site). Slowly penetrate the sclera-choroid complex to a needle depth of about 500 µm. In the pigmented rat, the needle end will 'disappear' beneath the pigmented choroid. For the brand of insulin needle used here, the distance from the needle tip to the bevel is 500 µm.
16. Carefully withdraw the insulin needle (a very small effusion of blood may be seen).
17. If excessive bleeding is noted, an eye spear can be applied to clear the hole, if necessary. Continued bleeding after the spear application indicates a vessel may have been damaged.
18. Guide the RPE cell-loaded injector needle into the pilot hole, with the opening facing down, and at an angle of about 10-15 degrees relative to the local surface of the sclera.
19. Gently insert the injector needle into the pilot hole to a depth of about 500 µm to access the subretinal space. There should be about a 100 µm margin between the edge of the black pen mark and the point where the needle is covered by the pigmented choroid (**Figure 1C** and **1D**).
20. Ask the assistant to gently depress the plunger of the injector syringe to inject the appropriate volume of cells (about 1.2 µL). Be ready to provide some counter-force as the assistant presses on the plunger.
    Note: Prior mock practice with the assistant can provide both the surgeon and assistant with the necessary experience with this step.
21. While visually focusing on the pen mark edge, hold the injector in place for 25-30 s and then slowly retract the injector. A small amount of backflow is commonly observed.
    Note: If no backflow is observed, there may have been an intravitreal injection. If you see backflow through the seal or cells filling beneath the sclera, the injection was too shallow.
22. Rinse the cell efflux from the injection site with the sterile eye wash 3 times and collect the excess with a cotton applicator.
23. Apply a drop of eye lubricant on the operated eye and transfer the rat to the OCT station to examine the location of transplanted cells and the size of the subretinal bleb.

3. Post-injection Treatment

1. **Anti-inflammatory and pain reliever treatment**
   1. Inject buprenorphine at 0.1 mg/kg body weight in saline subcutaneously to decrease pain.
   2. Inject dexamethasone at 1.6 mg/kg body weight in saline by intraperitoneal injection (I.P.) for inflammation control.

2. **Animal recovery**
   1. Return the rat to the recovery cage under a heat lamp to maintain body temperature.
   2. Observe the operated eye for signs of hemorrhage.
   3. Observe the rat to ensure it comes out of anesthesia.
   4. Return the recovered animal to a fresh cage and flag the cage with a surgery card, and monitor daily for any signs of distress, ocular hemorrhage, or corneal opacities. Notify the veterinarian immediately if there are concerns.

Representative Results

Using the technique described in this article, we consistently delivered hRPESC-RPE cells into the subretinal space of RCS rats by precisely controlling the location, angle, and depth of the injector needle inserting into the tissue (**Figure 1B**-**D**). Immediately following transplantation, an OCT examination was performed to observe the injection site and the subretinal bleb created by the transplanted cells. Post-surgical OCT evaluation serves as a screening tool for evaluating the quality of injections and monitoring for retinal damage or hemorrhage. Both the subretinal bleb (**Figure 2A**, **C** and **D**) and the injection site (**Figure 2A** and **B**) could be seen clearly under the OCT scanning. The subretinal bleb usually resolves in 24 hours after injection. Although measurement of the size of blebs is difficult using OCT, we can estimate the bleb area assuming it is equal to the photoreceptor preservation area by cell transplantation. We previously demonstrated that a 1 µL injection of 50,000 cells could result in saving about 6-7% area of the RCS retina around the injection site[13]. As shown in **Figure 2A**, **C** and **D**, the retinal layers were intact at the injection site, no blood was detected in the bleb, and no cells were observed in the vitreous, demonstrating minimal trauma caused by the injection. Additionally, representative OCT images of failed injections were also included for reference (**Figure 2E** and **F**).

With the use of OCT as a feedback tool, we optimized the angle and depth of injector needle insertion into the tissue. Once optimized, this method allowed us to achieve a success rate of 90.8% subretinal access with only 5.7% surgical fails, based on the results of more than 300 previous subretinal injections performed in our other studies[13] (**Table 1**). In the remaining 3.5%, the OCTs were not performed for several reasons, including eyes not in an adequate position due to isoflurane anesthesia-associated eye rolling[17].

At 7 days after transplantation, the operated rat eyes were enucleated, fixed and sectioned for immunohistological analysis. A human cell nuclear marker (Hunu)[18] and an RPE cell marker (OTX2)[19] were used to detect the transplanted cells. **Figure 3** showed a thick layer of transplanted RPE cells in the subretinal space that, one week after injection, was positively stained with both markers, confirming the identity and the successful delivery of transplants. At one week after injection, the large number of cells, as shown in **Figure 3C**, may rapidly decline to a small number later due to host immune response even in immunosuppressed RCS rats[20]. Nevertheless, as mentioned above, the degenerated photoreceptor layer of RCS rat eyes can be found to be rescued for at least 2 months after transplantation with hRPESC-RPE[13].

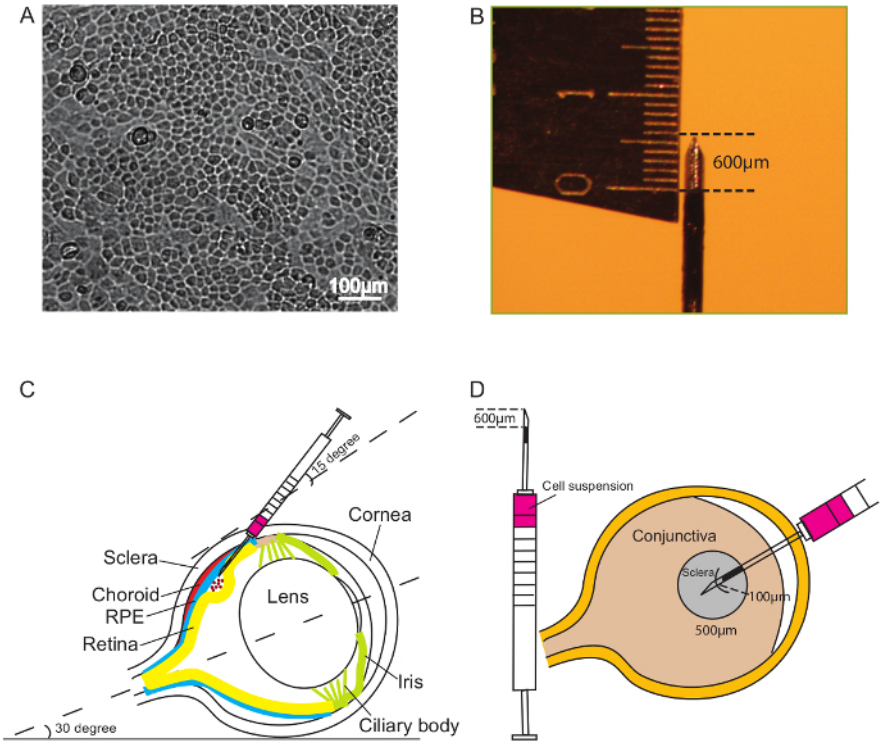

Figure 1: An image of 4-week old P2 hRPESC-RPE cells and a demonstration of the angle and depth that the injector needle uses during the injection. (A) A phase-contrast image of 4-week old P2 hRPESC-RPE cells used for injection. Scale bar = 100 µm. (B) A schematic showing the 600 µm distance between the edge of the marker and the tip of the injector needle measured by a microscale. The minimum graduation of the microscale is 100 µm. (C) A cartoon showing the cross-section of the anatomical structure of a rat eye and a side view of the angle and depth that the injector needle inserts into the eye wall. The pupillary axis of the rat eye is 30 degrees relative to the table top and the injector needle is 15 degrees relative to the local surface of the eyeball. (D) A cartoon showing the starting point of the marker on the injector needle and the top view of the injection site where 500 µm of the injector needle is inserted into the tissue and a 100 µm space is left between the opening of the injection hole and the edge of the marker. The location of the hole is 1,200-1,500 µm posterior to the limbus. The needle tip is shown on its side, but should be face-down during the injection. Please click here to view a larger version of this figure.

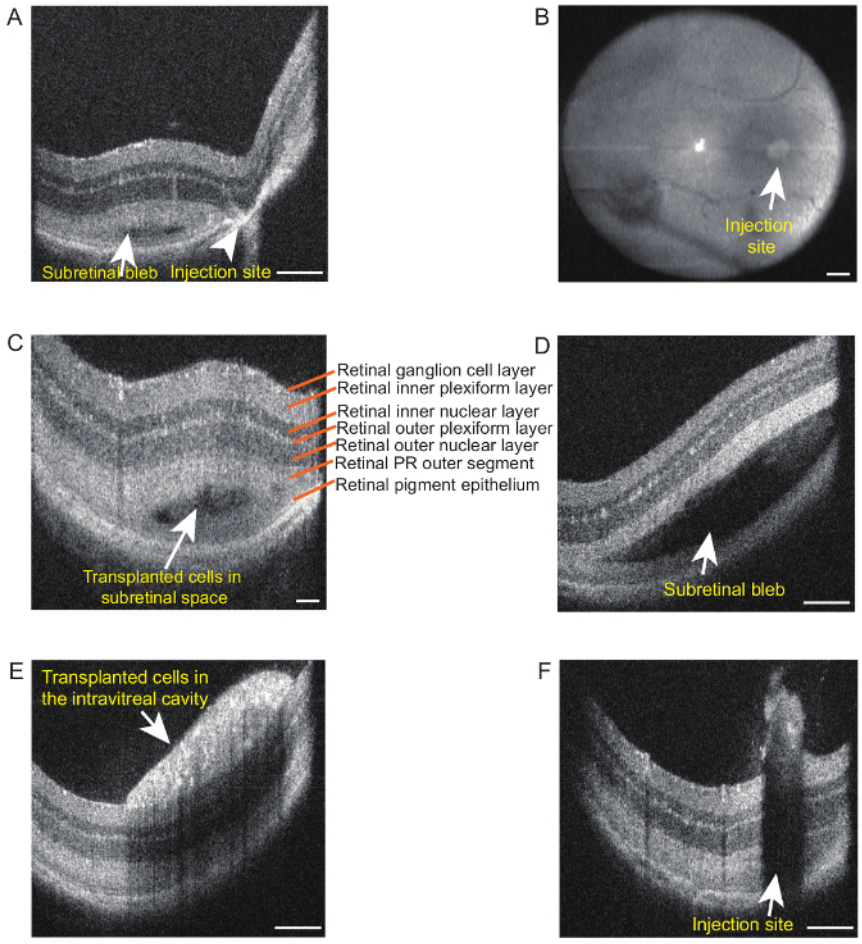

Figure 2: OCT images of the operated rat eye immediately after injection. (A) An OCT B-scan image of an operated eye showing a subretinal bleb and injection site, without intravitreal hemorrhage. (B) An OCT volume intensity projection (VIP) image of a B-scan series representing the enface fundus image of the injected area. The small injection site is visible in the VIP image showing the minimal trauma. (C) An enlarged OCT image of (A) showing the transplanted cells in the subretinal space with all retinal layers marked. This image demonstrated that the transplanted cells were located in the subretinal space. (D) An OCT B-scan image showing an average size subretinal bleb. (E) An OCT B-scan image showing a failed subretinal injection with CS located in the intravitreal space. (F) An OCT B-scan image showing a failed subretinal injection with the entire retina poking through at the injection site. Scale bars = 100 µm. Please click here to view a larger version of this figure.

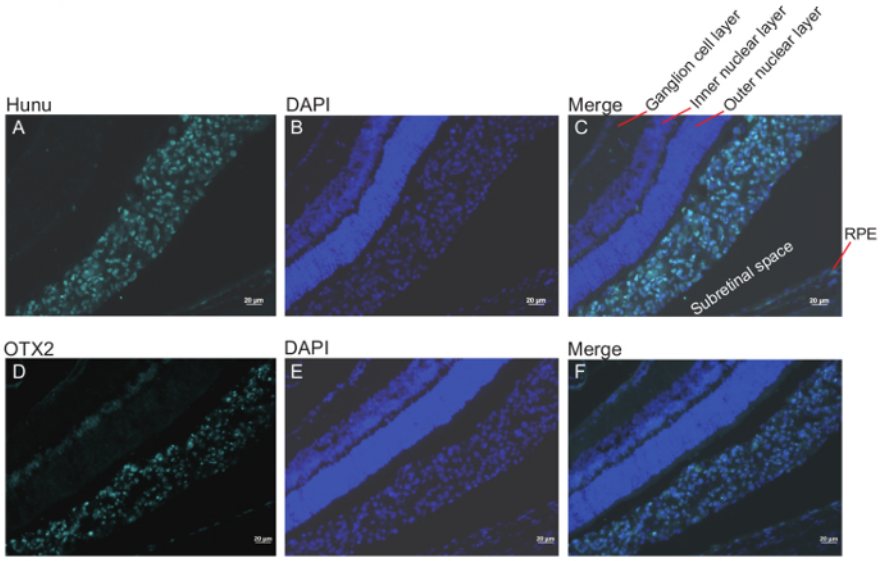

Figure 3: Immunohistological staining of retinal frozen sections after transplantation of hRPESC-RPE cells. (A) Human cell nuclear marker (Hunu) staining indicating the detection of transplanted human RPE cells. (B, E) Cell nuclear counter staining (4',6-diamidino-2-phenylindole; DAPI) showing the retinal layers, the transplant, and RPE layer. (C) A merged image of Hunu and DAPI indicating that the transplanted hRPE cells are located in the subretinal space. The separation between the transplant and RPE layer is a processing artifact associated with cryo-protecting RCS eyes for frozen sections. (D) RPE cell marker (OTX2) staining of the transplanted human RPE cells. (F) A merged image of OTX2 and DAPI. Scale bar = 20µm. Please click here to view a larger version of this figure.

	Total injected RCS rat eyes	Good subretinal blebs under OCT	Small subretinal blebs under OCT	Total non-complicated subretinal blebs under OCT	Complicatd subretinal blebs under OCT (i.e. air bubble in it or retinal hemorrhage)	No OCT performed	Surgical fails (no subretinal bleb under OCT)
Count	314	260	25	285	5	6	18
% of total injected eyes	------	82.80%	7.96%	90.76%	1.59%	1.91%	5.73%
(These data are summarized from ten cohorts of subretinal injections in RCS rats)							

Table 1: A summary of subretinal injections from ten experimental cohorts in RCS rats.

Discussion

The subretinal injection technique depicted in this article is via the trans-scleral pathway, where the injector needle penetrates the outer layers (sclera-choroid-RPE complex) of the eye wall without harming the neural retina or disturbing the vitreous cavity. An alternative trans-vitreal approach has a potential risk of lens damage leading to cataract, since rodents' lens occupies the majority of the vitreous cavity. Compared to this method, our technique is less risky and causes minimal trauma as the injector needle does not need to go across the entire vitreous cavity to reach the subretinal space. Indeed, OCT examinations in our studies showed very rare retinal penetration events and, in follow-up exams in animals, there are no persistent retinal detachments. Additionally, the injection site is very small (< 200 µm in diameter) when using a 33-gauge injector needle so the structural disturbance of the sclera-choroid-RPE complex is very limited. After needle retraction, the injection hole self-seals automatically so no stitch or tissue glue is needed.

Complications with the surgery include excessive bleeding around the injection area or from within the pilot hole. When using forceps to grip the conjunctiva margin at the limbus, only gentle force is needed to avoid pinching blood vessels and decrease possible bleeding. Before creating the pilot hole, examine the intended injection site to avoid penetration of superficial blood vessels. By using a spear, the pilot hole can be cleared of blood before insertion of the cell injection needle. If bleeding from the pilot hole persists after a few applications of the spear, a vessel may have been broken. Another complication we observed is a small percentage of RCS rats developing corneal opacities post-operatively. In some cases, the opacities were chronic and others were transient. Animals with persistent opacities were removed from the study group. Corneal opacities can develop due to eye dryness, physical damage, inflammation, drugs or chemical irritation[21]. To reduce their formation, the eye should be kept moist by maintaining ample eye lubrication, and avoid touching the cornea with a cotton applicator or other tools during the operation.

The injection protocol outlined here uses defined approach angles and needle depths relative to ocular landmarks, and eye angles relative to the surgical table in our set-up. The use of post-injection OCT scans was important in refining these injection parameters to provide reproducible control of RPE cell transplantation into the rats' subretinal space with high accuracy. Once mastered by repeated practice, the method is simple

to perform. It is recommended, especially in training, that post-surgical ocular exams are performed to determine outcomes. The angle and depth of the injection needle will likely need adjustment accordingly to this feedback depending on the surgical set-up, age of animal, and/or if other animal species are used (*e.g.*, mouse).

To evaluate the injection quality and determine inclusion or exclusion from a study, the presence of subretinal bled from OCT observation is critical. In laboratories, where OCT is unavailable, the criteria described below can be used for a quick screening by surgeons to exclude suspected injection fails: (1) Prior to injection: (a) Pilot hole is made too deep (*i.e.*, significant depth > 500 µm; for the brand of insulin needle used here, the distance from the needle tip to the middle of the bevel is 500 µm). (b) Pilot hole bleeds excessively (*i.e.*, bleeding cannot be stopped by applying force on the hole with eye spears). (c) The injection needle goes too deep (*i.e.*, significant depth > 500 µm or the marker on injection needle goes into the sclera/choroid/RPE complex tissue). (2) During injection: (a) Unable to maintain the injection needle in the pilot hole during injection. (b) Leakage immediately around the injection needle during injection. (c) The injection needle is pushed too deep (*i.e.*, the marker on the injection needle goes into the sclera/choroid/RPE complex tissue) as noticed by the surgeon during the injection. (d) Unable to maintain the injection needle in position for more than 5 seconds after the injection. (e) Excessive blood when retracting the injection needle. (f) No backflow/efflux seen after retraction of the injection needle when combined with step 2.

Taken together, with careful management of the needle location, angles, and depths, the trans-scleral subretinal injection technique is highly reliable, accurate, and can carry minimal surgical trauma. With all these benefits, this technique can be used not only for delivery of RPE cells, but also for other cell types, compounds or gene therapies.

Disclosures

The authors have nothing to disclose.

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