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New ex vivo model of corneal endothelial phacoemulsification injury and rescue therapy with mesenchymal stromal cell secretome

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Abstract

PURPOSE: To develop a reproducible ex vivo model of corneal endothelial cell injury using phacoemulsification in porcine eyes and evaluate the effects of mesenchymal stromal cell secretome in this injury model.

SETTING: Department of Ophthalmology, University of Illinois at Chicago, Chicago, Illinois, USA.

DESIGN: Experimental study.

METHODS: A corneal endothelial injury model was optimized using different powers and durations of ultrasound energy inside ex vivo porcine eyes. Conditioned media from corneal mesenchymal stem cells was collected under serum-free conditions from passages 4 to 6. Immediately after the phacoemulsification injury, the anterior chamber fluid was replaced with unconditioned media or conditioned media and incubated at 37°C for 4 hours. At the end, endothelial cell viability was evaluated using trypan blue staining and analyzed with ImageJ software.

RESULTS: Using specific parameters (50% power for 30 seconds), phacoemulsification inside fresh porcine eyes led to a consistent level of endothelial cell injury. Incubation with corneal mesenchymal stromal cell-conditioned media after the injury significantly reduced endothelial cells loss compared with unconditioned media (mean 1.29% ± 0.91% [SD] and 5.33% ± 3.24%, respectively, $P < .05$).

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CONCLUSIONS: Phacoemulsification inside fresh porcine eyes provided a reproducible model to study endothelial cell injury. Treatment with corneal mesenchymal stromal cell secretome after injury appeared to significantly enhance the survival of corneal endothelial cells. This might provide a new strategy for preventing corneal endothelial cell loss after phacoemulsification or other endothelial injuries. Further in vivo studies are necessary to determine the therapeutic potential.

Cataract is a leading cause of age-related loss of vision.^{1,2} Phacoemulsification using ultrasound (US) energy is the preferred and most efficient technique for cataract extraction. A major unwanted side effect of phacoemulsification is corneal endothelial cell loss. Corneal endothelial cell loss in cataract surgery can occur intraoperatively as well as postoperatively. Intraoperatively, the degree of endothelial injury is directly related to the amount of US energy used for surgery.^{2,3} Fortunately, in most patients with healthy endothelium, a limited degree of corneal endothelial cell loss does not have a major detrimental effect on overall corneal endothelial function. Although newer technologies have reduced US energy and hence reduced endothelial cell loss during phacoemulsification, corneal endothelial cell loss can still be clinically significant and can be caused by operator-dependent factors and patient-dependent factors, such as dense cataracts and shallow anterior chambers.⁴⁻⁶ Thus, despite advances, pseudophakic corneal edema remains a leading indication for endothelial keratoplasty.^{7,8}

In this study, we standardized an ex vivo model of corneal endothelial cell injury using phacoemulsification in porcine eyes and further evaluated the effect of corneal mesenchymal stromal cell secretome as a new therapy to reduce corneal endothelial cell loss.

MATERIALS AND METHODS

Endothelial Injury Model

Fresh whole porcine eyes were obtained from a local abattoir. After each eye was washed with sterile saline, the globes were stabilized with pins inside the orbital cavity of a Styrofoam mannequin head used for ophthalmic surgery practice. The globes and heads were placed under an operating microscope. A clear corneal incision was made using a 2.8 mm keratome (Alcon Laboratories, Inc.). An Infiniti Vision phacoemulsification system (Alcon Laboratories, Inc.) was used. The phacoemulsification handpiece was placed inside the anterior chamber in a bevel-up position along with continuous irrigation with a balanced salt solution. Different settings were used to determine the optimum the energy and duration for the injury model. Specifically, 30%, 40%, 50%, and 60% power was used for a duration of 20, 30, 40, 50, or 60 seconds. Similar settings were studied in excised porcine corneas placed endothelial side up in a petri dish. At least 3 eyes were used for the experiment under each setting to primarily assess the severity of injury as well as the reproducibility. Once the optimum parameters were determined (50% power for 30 seconds), 10 eyes per group had the procedure with this setting to further assess its reproducibility and to test the intervention.

Corneal Mesenchymal Stromal Cell Culture

Human corneal mesenchymal stromal cells were isolated and expanded as described before.⁹ In brief, the corneoscleral button obtained from healthy cadaver eyes (kindly provided by Eversight Eye Bank, Ann Arbor, Michigan, USA) were washed 5 times with phosphate-buffered saline (PBS) containing 2× antibiotic–antimycotic and 2× penicillin–streptomycin (Thermo Fisher Scientific, Inc.). After the central button was removed using a trephine, the limbus was cut into 3 segments, which were placed in 2.4 IU of protease (Dispase II, Thermo Fisher Scientific, Inc.) for 1 hour at 37°C. Intact epithelial sheets were then removed from the stroma. The limbal segments were cut into small pieces and used directly for explant culture in alpha Minimum Essential Medium (MEM) media supplemented with 10% fetal bovine serum, 1× L-glutamine, and 1× nonessential amino acid (NEAA) (all Corning, Inc.). Culture media were changed every other day, and cells were subcultured by brief digestion with a cell-dissociation reagent (TrypLE Express, Thermo Fisher Scientific, Inc.) when 80% confluent. Passages 4 to 6 were used for the experiments. All experiments were repeated with mesenchymal stromal cells from 3 donors.

Corneal Mesenchymal Stromal Cell Secretome

Corneal mesenchymal stromal cell–conditioned media was prepared as described previously.⁹ Briefly, on reaching 100% confluency in a T175 flask, the mesenchymal stromal cells were washed 3 times with 30 mL prewarmed PBS. The media was then changed to phenol red free alpha MEM media (25 mL for each T175 flask) supplemented with 1× L-glutamine, and 1× NEAA. The conditioned media was collected after 48 hours. The cells were trypsinized and counted at the same time. The conditioned media was centrifuged at 500g speed for 15 minutes to remove cells or debris. The supernatant was transferred to a new tube and used for experiments the same day or was kept at 4°C for up to 1 week.

Enzyme-Linked Immunosorbent Assay

Human tumor necrosis factor (TNF)–stimulated gene-6 (TSG-6) protein levels in the conditioned media were determined by enzyme-linked immunosorbent assay as described previously.¹⁰ The obtained values were normalized to total cell numbers.

Treatment with Corneal Mesenchymal Stromal Cell Secretome

Following the endothelial injury, the anterior chamber was reformed with unconditioned media (media without serum) or corneal mesenchymal stromal cell–conditioned media, and the incision was closed using a 7–0 silk suture. The eyes were then incubated at 37°C for 4 hours (Figure 1).

Evaluation of Endothelial Viability

Endothelial cell viability after each modality of phacoemulsification was evaluated following vital staining using trypan blue and alizarin red, and the response to treatment was evaluated after vital staining using trypan blue only, as described previously.¹¹ After at least 10 photographs were taken from random locations at ×5 magnification using a Zeiss Axioskop 2 Plus microscope (Carl Zeiss Meditec AG), ImageJ software^A was used to quantify the dead cells areas and provide the ratio of the dead areas to the whole selected

areas. The results were analyzed using a 2-sided unpaired *t* test with Excel software (Microsoft Corp.) and presented as the mean $\pm$ SD. The level of significance set at a *P* value less than 0.05.

RESULTS

Ex Vivo Phacoemulsification Results in Reproducible Endothelial Injury

An ex vivo porcine model of corneal endothelial cell injury using phacoemulsification was standardized (Figure 1). Using fresh-cut porcine corneas provided very inconsistent results because of floating of the corneas and their movement in the dish (resulting from US energy). Using fresh porcine whole eyes ex vivo at different US energies and durations (using at least 3 eyes for each setting) achieved different levels of corneal endothelial injury. A 30-second exposure to 30% power resulted in very mild injury. Although 30% power with duration of 60 seconds or more induced significant injury, the injuries were variable and inconsistent in several experiments. A similar effect was observed with 40% power; thus, less than 50% power provided minimal injury with lower exposure times and inconsistent injury with higher exposure times. Furthermore, 60% power resulted in severe damage to the endothelium. The best and most reproducible method was 30 seconds of exposure to the phacoemulsification probe inside the anterior chamber in a bevel-up position with 50% power (along with continuous irrigation with a balanced salt solution). This setting was further evaluated using 10 additional eyes. This setting induced moderate injury that was significant but not excessive. The degree of injury was also consistent in these eyes (mean endothelial cell loss 5.33%; 95% confidence interval, 3.32–7.34). Figure 2 shows representative images of different levels of corneal endothelial damage using different modalities.

Exposure to Corneal Mesenchymal Stromal Cell Secretome Protects Endothelial Cells After Phacoemulsification Injury

Figure 3, *A* and *B*, show representative photographs of the trypan blue staining of the corneal endothelium treated with unconditioned media or corneal mesenchymal stromal cell-conditioned media. Figure 3, *C*, shows the results of analysis of endothelial cell loss after the procedure and treatments. As seen in the figures, incubation with corneal mesenchymal stromal cell-conditioned media significantly reduced endothelial cells loss after phacoemulsification (mean $1.29\% \pm 0.91\%$) compared with unconditioned media (mean $5.33\% \pm 3.24\%$) ($P < .05$).

Corneal Mesenchymal Stromal Cell Secretome Containing Tumor Necrosis Factor—Stimulated Gene-6

The concentration of a well-known therapeutic factor secreted by mesenchymal stromal cells, TNF- α stimulated gene/protein (TSG-6), was measured in the corneal mesenchymal stromal cell secretome. The corneal mesenchymal stromal cell secretome contained considerable amounts of TSG-6 compared with unconditioned media ($P < .01$).

DISCUSSION

The corneal endothelium is critical for maintaining corneal transparency and function.^{12,13} The corneal endothelial cells perform this role by keeping the corneal stroma in a state of constant hydration via 2 major mechanisms; that is, the active fluid pump and barrier function.¹³ There is an age-related decline in the number of endothelial cells in humans.^{14–16} The corneal endothelial cell density (ECD) typically starts with 4000 cells/mm² at birth and gradually decreases with age, with the average being around 2500 cells/mm² in adults and falling below 2000 cells/mm² in the elderly.^{12,17–19} Corneas with an ECD below 1000 cells/mm² might not tolerate intraocular surgery, and corneal edema and decompensation usually occurs when the ECD falls below 500 cells/mm².^{16–20}

It is well known that after injuries, the corneal endothelium cannot regenerate. The repair process involves enlargement of the residual cells, amitotic nucleus division, migration, and rosette phenomenon, which together ultimately result in a reduction in ECD, an increase in mean size of the cells, and disruption of the normal hexagonal cell pattern.^{17,20}

Corneal endothelial cell loss is a major side effect of phacoemulsification, resulting from heat and free radical formation by US waves during the procedure.^{6,21–23} Several studies^{24–27} report endothelial cell loss between 8.0% and 16.7% as a side effect of this surgery. Since the introduction of phacoemulsification, significant advances have been made to minimize corneal endothelial cell loss.¹⁷

At present, the only available choice for treating corneal endothelial dysfunction is corneal endothelial transplantation.^{7,8,28} A possible alternative could be to use strategies that save partially damaged endothelial cells from apoptosis and help promote their repair and regeneration.

Mesenchymal stromal cells play an important role in tissue repair and maintenance and are widely studied for cell-based therapies.^{9,29,30} Recently, corneal mesenchymal stromal cells were shown to be suitable for therapeutic applications in the cornea.⁹ Previous studies^{9,31,32} found that the therapeutic effects of mesenchymal stromal cells are mainly mediated through their secreted factors. Tumor necrosis factor- α stimulated gene/protein is among these secreted factors.^{33,34} It is released by mesenchymal stromal cells in response to inflammation and has the beneficial effects of mesenchymal stromal cells in the treatment of heart,³⁵ cornea,³⁶ brain,³⁷ and lung diseases.³⁸ Specifically, it has been shown that TSG-6 protects corneal endothelial cells from transcorneal cryoinjury through suppression of inflammation.³⁹ Our results show that conditioned media derived from corneal mesenchymal stromal cells contains considerable amounts of TSG-6 protein; thus, the observed protective effects against US-induced corneal endothelial cell damage could be partially attributed to this secreted factor.

Stanniocalcin-1 (STC-1) is another important factor secreted by mesenchymal stromal cells.^{33,34} It has also been shown to protect cells from oxidative damage and is a well-known antiapoptotic agent.^{40,41} Stanniocalcin-1 is known to be released by mesenchymal stromal cells in response to apoptotic cells' signals.⁴² Stanniocalcin-1 has also been shown to have protective effects against ischemia⁴³ as well as antiinflammatory effects.⁴⁴ Although we did

not measure this specific factor in our mesenchymal stromal cell—conditioned media, we hypothesize that it might also play a role in the observed effect. The potential role of STC-1 as well as other factors can be further evaluated in future studies.

Another new aspect of this study is the use of an ex vivo phacoemulsification injury model. Various methods have been used to induce corneal endothelial cell injury, including chemical injury,⁴⁵ cryoinjury,³⁹ and transplantation of mechanically injured corneas.⁴⁶ The main advantage of our injury model over these models is that it is more similar to what happens in patients clinically. Although we chose to use settings that induce significant, but not severe injury, our model has the potential to be modified depending on the desired level of injury. One disadvantage of this model is that it is performed ex vivo; thus, other factors such as aqueous humor are not present. However, the only alternative is to use in vivo models which, given regulatory approvals, adds significant cost and delays the performance of the studies.

In conclusion, we have introduced a new and reproducible ex vivo model of corneal endothelial cell injury using phacoemulsification. We propose that corneal mesenchymal stromal cell secretome could provide a new strategy to prevent corneal endothelial cell loss after phacoemulsification. In vivo studies are necessary to determine its true therapeutic potential. Also, further studies are needed to define more precisely the active ingredients of the corneal mesenchymal stromal cell secretome.

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WHAT WAS KNOWN

- Most studies of phacoemulsification corneal endothelial injury involve in vivo models. Other injury models such as cryoinjury do not represent a common clinical scenario in patients.
- At present, the use of intraoperative strategies to reduce corneal endothelial cell loss is the main approach that is available.

WHAT THIS PAPER ADDS

- This new and reproducible ex vivo model of corneal endothelial cell injury using phacoemulsification (US) reduces the need for more expensive and cumbersome in vivo models.
- The new method uses using secreted factors from mesenchymal stem cells to treat and prevent corneal endothelial cell loss. These factors are known to have antiapoptotic effects.

A new reproducible ex vivo model of corneal endothelial cell injury using phacoemulsification showed that mesenchymal stromal cell–conditioned secretome could provide a new strategy to prevent corneal endothelial cell loss.

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Figure 1.

Ex vivo porcine corneal endothelium injury model using phacoemulsification. *A*: A clear cornea incision was made using a 2.8 mm keratome. *B*: The phacoemulsification handpiece was placed inside the anterior chamber in a bevel-up position, and eyes had 30 seconds of exposure with 50% power along with continuous irrigation with a balanced salt solution. *C*: After the injury, the anterior chamber was reformed with unconditioned media or corneal mesenchymal stromal cell-conditioned media. *D*: The incision wound was closed using a silk 7-0 suture.

Figure 2.

A: Very mild injury was observed with 30 seconds exposure to 40% power. This amount of injury was not significant for the purpose of this study. *B:* The best injury model was achieved with 30 seconds exposure to 50% power, which created a significant, but not too severe injury. *C:* Thirty seconds exposure to 60% power. *D:* Sixty seconds exposure to 60% power. Both resulted in a very severe endothelial injury (scale bars = 100 μ M).

Figure 3.

Vital staining of corneal endothelium and analysis of corneal endothelial cell loss following different treatments. Vital staining of corneal endothelium treated with unconditioned media (A) and corneal mesenchymal stromal cell-conditioned media (A) after phacoemulsification injury (scale bars = 100 μM). C: Incubation with corneal mesenchymal stromal cell-conditioned media significantly reduced endothelial cell loss compared to unconditioned media (n = 10; **P* < .05). D: Enzyme-linked immunosorbent assay confirmed that corneal mesenchymal stromal cell-conditioned media contained considerable amounts of TSG-6 compared with unconditioned media (mean 658.68 ± 202.54 pg/mL versus 1.81 ± 1.53 pg/mL, respectively; n = 4; **P* < .01) (Co-MSC = corneal mesenchymal stromal cell-conditioned; TSG-6 = human tumor necrosis factor—stimulated gene-6).