

Isolation efficiency of collagenase and EDTA for the culture of corneal endothelial cells

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Purpose: Tissue engineering of the corneal endothelium, as well as cell therapy, has been proposed as an alternative approach for the treatment of corneal endotheliopathies. These approaches require in vitro amplification of functional corneal endothelial cells (CECs). The goal of this study was to compare two common isolation methods, collagenase A and EDTA (EDTA), and determine whether they influence cell viability, morphology, and barrier function.

Methods: Human eye bank research-grade corneas were used to isolate and cultivate CECs. All donors were more than 40 years old. Two Descemet membranes from the same donor were used separately to compare the collagenase A and EDTA cell isolation methods. The number of isolated cells, cell viability, morphology, and barrier functionality were compared.

Results: A higher isolation efficiency of viable CECs and a higher circularity index (endothelial morphology) were obtained using collagenase A. Passage 3 cells presented similar barrier functionalities regardless of the isolation method.

Conclusions: This study showed that isolation of CECs using collagenase A yields higher isolation efficiency than EDTA, delaying the loss of endothelial morphology for early passage cells.

The corneal endothelium covers the inner side of the cornea, facing the anterior chamber of the eye, which contains aqueous humor. This thin monolayer of hexagonal cells forms a leaky barrier, allowing aqueous humor to enter the corneal stroma. To maintain stromal deturgescence, endothelial cells pump fluid out of the stroma using ionic pumps and cotransporters. This pump–leak balance is essential for corneal transparency [1].

Trauma or pathology can cause endothelial dysfunctions in which corneal endothelial cells (CECs) are unable to maintain the pump–leak balance, resulting in stromal edema and vision loss [2]. Currently, graft transplantation is the only clinical treatment to restore vision for patients with endotheliopathies. The patient's decompensated corneal endothelium is replaced by transplanting a Descemet membrane (with the attached corneal endothelium) from a healthy cadaveric eye bank cornea. To that end, surgeons perform endothelial keratoplasty (EK) procedures, such as Descemet stripping automated endothelial keratoplasty (DSAEK) or Descemet membrane endothelial keratoplasty (DMEK). Both procedures are safe and provide good clinical outcomes [3]. One

limitation of these procedures is that a single donor cornea can treat only one patient's eye.

As an alternative to native tissues, we developed a tissue-engineered corneal endothelium by seeding cultured CECs on devitalized corneas [4]. The cultured cells reformed a functional corneal endothelium [5,6]. This tissue engineering approach, also proposed by others [7-11], could be used in EK procedures. Another proposed alternative treatment consists of injecting cultured CECs into the anterior chamber of the patient's eye. This approach is currently in clinical trials with good clinical outcomes [12]. Whether cultured cells are used for tissue engineering or cell injection, the expansion of CECs in vitro has the major advantage of increasing the number of cells and thus, the number of patients who can be treated using a single donor. Cell culture is key to the success of these alternative treatments.

Donor age impacts proliferation capacity and endothelial phenotype in vitro. It is known that CECs from older donors have lower proliferative rates and may generate cells with a nonfunctional phenotype [13]. Despite their lower proliferative capacity, CECs from older donors can be expanded when using the appropriate culture method [14-16]. The endothelial cell density (ECD) of donor corneas decreases with age [17-24]. Of course, high ECD allows for more cells to be isolated, which favors the successful culture of CECs, as

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lower cell density is linked to decreased proliferative capacity [25]. However, most of the donor corneas available for cell isolation come from older donors. Thus, it seems important to be able to isolate the maximum number of cells from these older corneas to be able to generate a high number of functional CECs.

An important parameter of CEC culture success is the cell isolation method, as it is the first step in CEC culture. A study demonstrated that the isolation method influences the cell attachment capacity of the isolated CECs, resulting in a higher rate of culture success, as collagenase A was more successful in starting CEC cultures than 0.1% dispase or 0.05% trypsin/EDTA [26]. This result is consistent with another paper that showed collagenase A digestion is better than dispase II for the isolation of human corneal endothelial cells based on the quality of the cell–cell junctions of the cultured cells and the preservation of the basement membrane component [27]. Our laboratory [6,28-34], as well as others [35-41], uses Dr. Joyce's technique for isolating CECs [13], which consists of a gentle dissociation of endothelial cells from the Descemet membrane using EDTA. However, in recent years, the collagenase A method has gained popularity [27,42,43]. Therefore, the aim of this study was to compare these two isolation techniques (collagenase A and EDTA isolation) in terms of the number of isolated viable cells, proliferation capacity, CEC morphology, and functional barrier integrity before switching to the collagenase method.

METHODS

This study was conducted according to our institutions' guidelines and the Declaration of Helsinki. The research protocol was approved by the "Bureau de l'éthique de la recherche du CHU de Québec – Université Laval" ethics committee (DR-002-1382) and adhered to the ARVO statement on human subjects. Twenty-seven pairs of cadaveric corneas (see Appendix 1 for a precise description of the specimens and their use), unsuitable for human transplantation, were obtained from a local eye bank (Centre Universitaire d'Ophthalmologie (CUO) Eye Bank, Québec, Canada). Next of kin consent was obtained from Hema-Quebec for all the tissues provided for research.

Cell populations, cell isolation, and cell culture: Pairs of cadaveric corneas ($n = 27$, donor age range: 42 to 84 years old, mean \pm SD: 69 ± 10 , Appendix 1) were used to compare the two isolation methods. For each donor, one Descemet membrane was incubated at 37 °C in collagenase A (1 mg/ml, Sigma, Oakville, Canada) for 2–4 h, and the other was incubated at 37 °C in EDTA (0.02%, Sigma) for 45 min, followed by gentle pipetting for cell–matrix dissociation. CECs were seeded

on FNC coating mix ® (a proprietary blend that contains albumin, fibronectin and collagen)-coated plates (AthenaES, MJS Biolynx, Inc., Brockville, Canada) and cultured in Opti-MEM (Life Technologies, Burlington, Canada) supplemented with 8% fetal bovine serum (Hyclone, Logan, UT), 5 ng/ml epidermal growth factor (Austral Biologicals, San Ramon, CA), 0.08% chondroitin sulfate (Sigma), 20 µg/ml ascorbic acid (Sigma), and penicillin/streptomycin (Corning, NY). At confluence, CECs from both isolation methods were passaged with trypsin (0.05%)-EDTA (0.53 mM; Corning) and seeded at a density of 20,000 cells/cm². The CECs were expanded until P3. At P3, the CECs were further maintained at confluence for a week to stabilize the phenotype.

Isolation efficiency: To determine isolation efficiency, we used corneas with known ECD (provided by the eye bank; see Appendix 1). The center of the corneas was cut out using a biopsy punch (7.5 mm diameter). The theoretical initial cell quantity within that cut tissue was calculated to serve as the total cells' reference value (theoretical cell count = $3.75^2\pi \times$ ECD). Each central cornea was digested with EDTA or collagenase A, as described above. Aggregates were dissociated with a short trypsin-EDTA incubation to count the isolated cells using a hemacytometer. Cells were counterstained with trypan blue coloration to assess mortality immediately following isolation from Descemet membranes before cell seeding (% of dead cells; donor age = 70 ± 5.0 years old). Isolation efficiency (% of isolated cells) was calculated by the ratio of the number of isolated cells and the theoretical initial cell quantity (donor age = 70 ± 5.0 years old). Cell viability 2 h after cell seeding was also assessed using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) cell viability assay (Promega, Madison, WI; donor age \pm SD = 77 ± 8.0 years old). The MTS assay absorbance results were normalized to the EDTA condition value for each population (relative viability).

Immunostaining: Immunofluorescence analysis was performed on P0 or P3 cells cultured on FNC-coated glass coverslips (AthenaES). The 1-week postconfluent CECs were fixed with 4% paraformaldehyde (EMS, Hatfield, PA) for 10 min at room temperature. Cells were permeabilized with 0.2% Triton X-100 (Fisher Scientific, Ottawa, Canada) for 10 min before blocking with 1% bovine serum albumin (BSA; Sigma) diluted in PBS (1X; 137 mM NaCl, 2.7 mM KCl, 6.5 mM Na₂PO₄, 1.5 KH₂PO₄, 0.9 mM CaCl₂·2H₂O, 2.4 mM MgCl₂·6H₂O) for 1 h. Samples were labeled with the primary antibody directed against ZO-1 (mouse monoclonal; Thermo Fisher Scientific, Burlington, Canada), Ki67 (mouse monoclonal; BD Biosciences, San Jose, CA), or N-cadherin (mouse monoclonal; Dako, Agilent Technologies,

Mississauga, Canada) for 1 h at room temperature. The cells were rinsed and then incubated with the secondary antibody (anti-mouse immunoglobulin type G [IgG] Alexa Fluor 594; Invitrogen, 1/400), Hoechst 33,258 (Sigma, 1/100), and phalloidin 488 (Invitrogen, 1/200) for 1 h in the dark. Coverslips were mounted on glass slides with mounting media and kept at 4 °C until observation. Micrographs were acquired using a confocal microscope (LSM-800, Zeiss, Toronto, Canada) and Zen 2.3 system software (Zeiss). Ki67 relative expression (donor age = 65±11 years old) was determined from immunostaining images by measuring the area recovered by Ki67 and reporting it to the area recovered by Hoechst (nuclei).

Morphology assessment: Morphology was assessed by calculating the cell circularity index. Randomly selected cells from the phase-contrast images (n = 3 images/cell population) of confluent CECs were used to measure cellular circularity (n = 4 images/condition/donor; 50 cells per condition). Cell area and perimeter were measured with ImageJ software (National Institutes of Health and the Laboratory for Optical and Computational Instrumentation, University of Wisconsin, WI), and then circularity was determined with the following formula: $\text{Circularity index} = (4\pi \times \text{Area}) / \text{Perimeter}^2$. Hexagonal cells, a hallmark of endothelial morphology, have a circularity index of 0.87, and cells with fibroblastic morphology have a score closer to 0.

Transendothelial electric resistance (TEER): At passage 3, CECs were seeded on semipermeable 60 mm² filter inserts (EMD-Millipore, Etobicoke, Canada; n = 6; donor age = 60±10 years old) coated with the FNC-coating mix (AthenaES, Baltimore, MD). First, the medium was changed, and then TEER (Millicell ERS-2 voltohmmeter; EMD-Millipore) was measured after letting the fresh medium equilibrate for at least 30 min. During the TEER measurements, the CEC cultures were kept at 37 °C on a warming plate. Three measurements per insert were made, and three inserts were used per condition. Inserts without cells containing only the medium were used as blanks to normalize the measurements. Ten TEER measurements were taken after every medium change.

Statistical analyses: The results are presented as the mean of all measurements and standard deviation (SD). Statistical significance was calculated with ANOVA and a paired Student *t* test with GraphPad Prism 7. A *p* value of less than 0.05 was considered statistically significant.

RESULTS

Comparison of isolation methods at passage 0: As the isolation method can influence the quantity of seeded cells and their morphology, we first compared two isolation methods,

the classic EDTA method and the collagenase A method, on P0 CECs. We compared the two isolation methods using pairs of corneas from the same donors.

As shown in Figure 1A, collagenase A isolation produced a higher viability rate (EDTA = 1.0±0.7, collagenase A = 3.1±1.9, *p* = 0.03), which can be explained by the higher number of isolated cells (isolation efficiency). As shown in Figure 1B, a lower percentage of isolated cells was obtained using EDTA (40.5%±5.40) than collagenase A (54.4%±7.45). Furthermore, more dead cells were obtained using EDTA (70.5%±6.80; Figure 1C) than collagenase A (26.0%±4.90). We also investigated proliferation capacity with Ki67 immunostaining. Cells in both conditions showed the same relative expression of positive Ki67-expressing cells (Figure 1D,E).

Collagenase A isolation initially generated small cell aggregates, as shown in Figure 1F, while EDTA-isolated cells were individually dispersed. After 7 days of culture, CECs isolated with collagenase A were less fibroblastic and smaller than EDTA-isolated CECs (Figure 1F). The circularity index was higher for confluent P0 CECs isolated with collagenase A (Figure 2B; 0.67±0.07 for EDTA; 0.80±0.03 for collagenase A, *p* = 0.01).

Cell–cell junctions play an essential role in the corneal endothelium barrier function. We next compared the expression of the adherens junction protein N-cadherin and the tight junction protein ZO-1 at 7 days after seeding. Collagenase A–isolated CECs had more defined junctions, as N-cadherin and ZO-1 staining was strongly expressed at cell–cell borders (Figure 1G). The collagenase A condition also had well-formed actin rings at their apical side, and had fewer stress fibers, than the EDTA-isolated CECs (Figure 1G).

Comparison of initial isolation method throughout cell passages: Maintaining a functional phenotype throughout cell expansion is also an important parameter for successful cell therapies. Following EDTA or collagenase treatment, cells were passaged using trypsin/EDTA, seeded, and cultivated up to passage 3 (the isolation methods followed the same expansion protocol). At the third passage, the CECs were kept postconfluent for a week before the morphology and functionality assessments. CECs adhered to and generated a culture using both approaches. Cell morphology was evaluated using phase-contrast images of confluent cultures. As shown in Figure 2A,B, at P0 and P3, CECs isolated with EDTA had a lower circularity index than collagenase A (P0: 0.67±0.07 for EDTA and 0.81±0.03 for collagenase, *p*=0.01; P3: 0.59±0.04 for EDTA and 0.71±0.05 for collagenase, *p* = 0.02). The P1 and P2 cells had similar circularity indexes regardless of the isolation method.

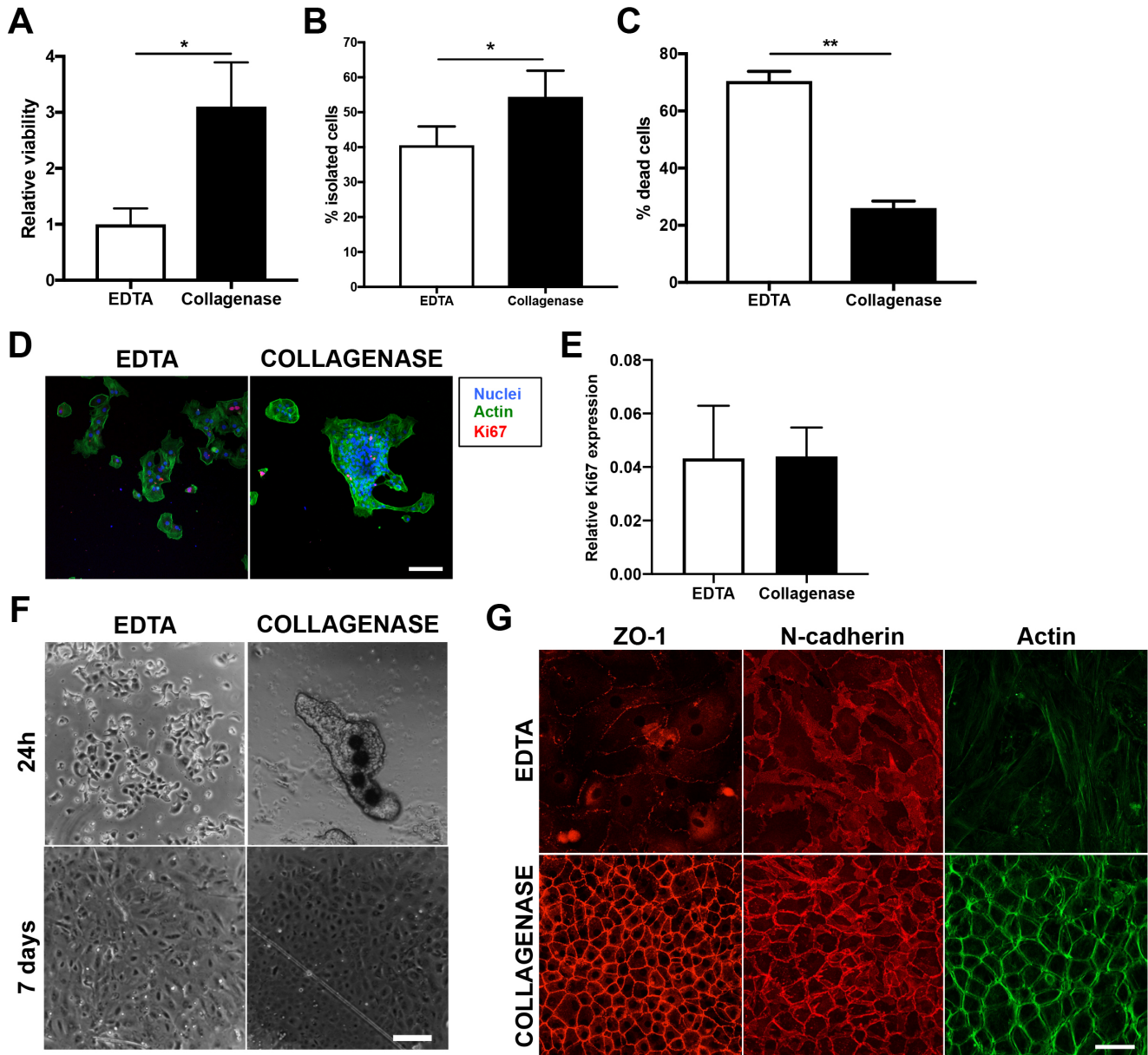


Figure 1. Primary (P0) cultures of corneal endothelial cells isolated with EDTA or collagenase. **A**: Cell viability, 2 h after cell isolation, relative to the EDTA condition. $n = 6$. **B**: Percentage of cells isolated from native Descemet membranes. $n = 4$. **C**: Percentage of dead cells immediately following cell isolation from Descemet membranes. $n = 4$. **D**: Representative images of indirect immunofluorescence staining of Ki67 (red), actin (green), and nuclei (blue) at 24 h postseeding. Scale bar = 100 μm . **E**: Relative expression of Ki67-expressing cells (reported on nuclei). $n = 3$. **F**: Phase-contrast images of P0-cultured corneal endothelial cells at 24 h and 7 days postseeding. Scale bar = 200 μm . **G**: Representative images of indirect immunofluorescence staining of ZO-1 (red), N-cadherin (red), and actin (green) at 7 days postseeding. Scale bar = 50 μm . Results are expressed as mean \pm standard error of the mean (SEM). Two-way ANOVA was performed with GraphPad Prism 7. * $p \leq 0.05$.

TEER was used to measure endothelial barrier integrity because a low TEER is associated with a loss of barrier function [44,45]. There was no significant difference in TEER values between CECs isolated using EDTA or collagenase A

at any of the time points (Figure 2C) or after the tenth media change (Figure 2D).

CECs were also seeded on FNC-coated glass coverslips and immunostained for the tight junction protein ZO-1. Overall, ZO-1 expression was similar regardless of the

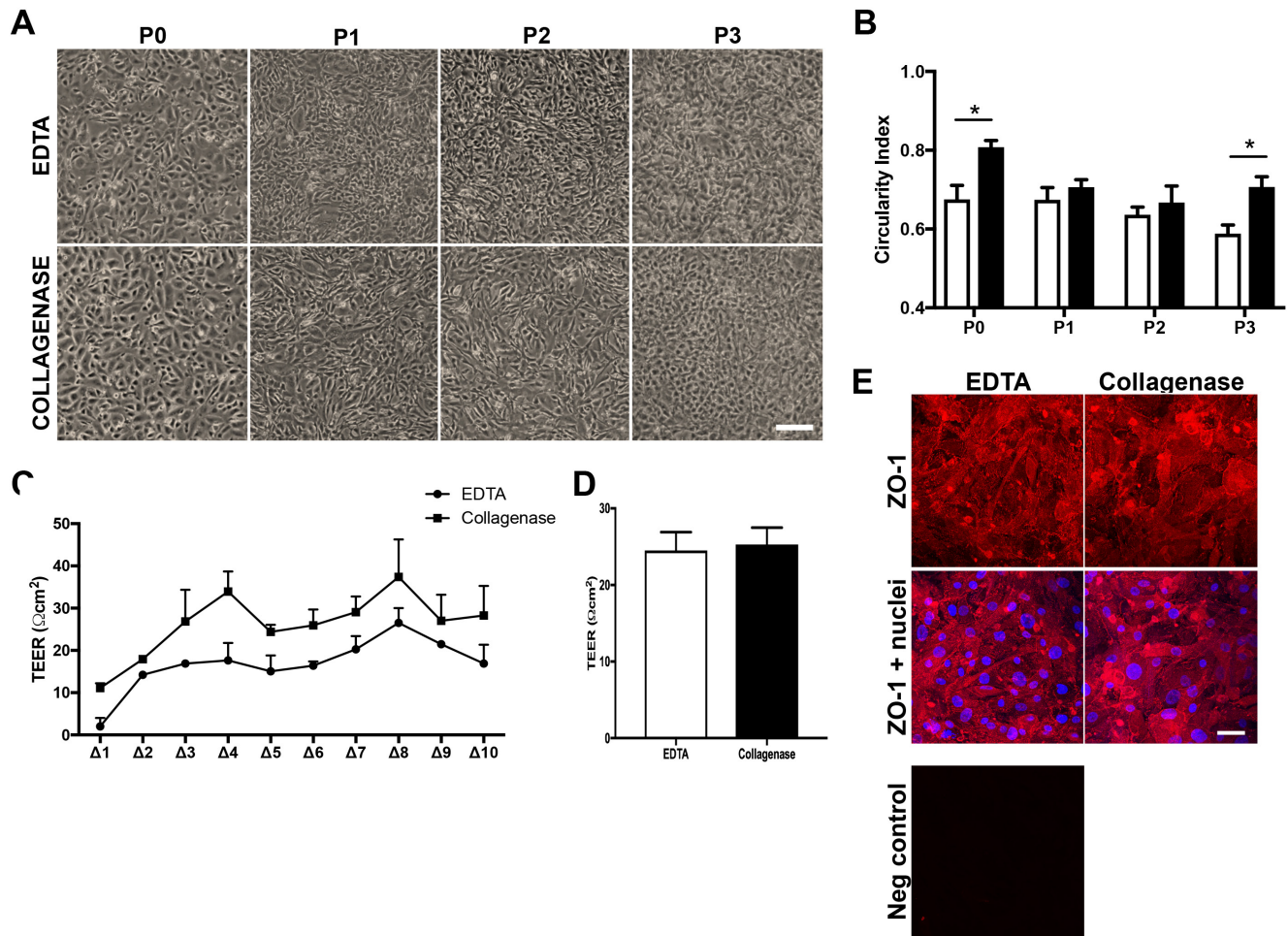


Figure 2. Cell functionality throughout the cell expansion of corneal endothelial cells isolated with EDTA or collagenase A. **A:** Phase-contrast images of cultured corneal endothelial cells throughout cell expansion. n = 4. Donor age ± standard deviation (SD) = 55±11 years old. **B:** Circularity index of cultured corneal endothelial cells throughout cell expansion. n = 4; 100 cells (four images) per condition per population were measured. White bars = EDTA, black bars = collagenase A. **C:** Transendothelial electrical resistance (TEER) measurements throughout time. TEER was measured for ten media changes (Δ; media changes were performed every 2 days). Mean ± Standard error of the mean (SEM) of one representative cell population. **D:** TEER measurements at the final media change, where nine measures per population per condition were taken. Mean ± SEM of four different cell populations. Two-way ANOVA was performed with GraphPad Prism 7. *p≤0.05. **E:** Indirect immunofluorescent staining of ZO-1 (red). Cell nuclei were counterstained with Hoechst (blue). Negative control (primary antibody omitted). n = 4. Scale bar = 50 μm.

isolation method used at P3, as both conditions exhibited scattered cytoplasmic ZO-1 (Figure 2E).

DISCUSSION

This study compared two common isolation methods to identify which one generated a successful cell culture of CECs from donors more than 40 years old. The results demonstrated that collagenase A isolation generated more viable P0 CECs that expressed junction-related proteins than EDTA, showing that collagenase A should become the technique of choice over EDTA for isolating CECs. However, the benefits

of collagenase isolation were lost after serial passages in trypsin-EDTA. Interestingly, P3 trypsin-passaged CECs, whether initially isolated using EDTA or collagenase, did not form tight junctions as quickly as in P0, as demonstrated by the cytoplasmic expression of ZO-1 after 7 days of postconfluency, as well as similar TEER values.

To the best of our knowledge, this study is the first to compare the isolation of CECs using EDTA and collagenase A for corneas from donors more than 40 years old. Both treatments have advantages and drawbacks. EDTA is a nonenzymatic approach that chelates calcium and magnesium,

inducing misconformation of cadherins and disrupting lateral junctions [46]. Following the EDTA treatment, CEC can be detached from the Descemet membrane using light mechanical stress. However, pipetting cells up and down can generate cell mortality by physically damaging the cells, as well as anoikis [47], programmed cell death, which occurs when cells are no longer attached to their basal membrane. This could explain the high cell mortality rate. As an advantage, it has been shown that disruption of cell junctions activates proliferation by releasing sequestered transcription activators such as β -catenin and ZONAB, known to promote cell proliferation [48,49]. As the amplification of CECs is the main goal of culturing them, it is of interest to unlock the mitotic block caused by mature junctions observed on the native corneal endothelium. Zhu et al. (2012) previously showed that nuclear p120 can restart the cell cycle without disrupting cell–cell junctions [48]. On the other hand, collagenase A acts by digesting collagen bounds that form the Descemet membrane. Extracellular matrix digestion releases cells without junction disengagement, and it appears to advantage CEC culture by delaying endothelial–mesenchymal transition [50] while inducing cell proliferation as Ki67 staining showed (Figure 1D). Small cell aggregates obtained with collagenase A allow for more cells to attach to the culture plate; however, they affect cell distribution. To avoid clumps of cells adhering to the cell culture plasticware, some research teams have added a brief trypsin step before cell seeding [27,51].

Of course, cell isolation is only the first step, and many other variables influence CEC expansion, such as the coating on which cells are seeded (FNC [13,52,53], type IV collagen [52,53], laminin [54,55]), the initial seeding cell density [25], the culture medium [35,56–58], and the adjustment of the culture medium composition according to their proliferating or maturing states [28,59,60]. Low mitotic agents in culture media also delay CEC senescence during cell expansion [61]. With an optimal combination for CEC culture, a maximum of high-quality cells could be generated from cadaveric pairs of corneas, even from donors who are now discarded because of their age.

In summary, this study showed that CEC isolation using collagenase A would be optimal for future cell therapies, as it yields higher morphology and a higher number of viable isolated cells at P0. Furthermore, P3 CECs isolated with collagenase A had an increased endothelial phenotype and similar barrier functionality as the same cells isolated using EDTA. These results were obtained using older donors, which is encouraging for the availability of corneas suitable for expanding functional CECs. Therefore, collagenase A will be used in our subsequent studies. This study is a small step

toward obtaining clinical-grade CECs for the treatment of endotheliopathies.

APPENDIX 1. TISSUE DONORS DETAILS

To access the data, click or select the words “[Appendix 1.](#)”

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