Transplantation of cultured corneal endothelial cells: Towards clinical application

Spinozzi D.

CHAPTER 4

Effect of GMP-compliant collagenase on the *in vitro* isolation and expansion of human corneal endothelial cells

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Article in preparation.

ABSTRACT

Purpose: To compare the effects of two different collagenases on human corneal endothelial cells (hCEC) isolation to potentially improve the culture protocol for research-grade corneas from elderly donors.

Materials and methods: Pre-screening included testing of enzymatic activity of a GMP-compliant collagenase (Nordmark) and a research-grade collagenase A (Sigma Aldrich) by colorimetric assays and cellular viability after collagenase exposure was assessed using human amniotic membranes. Primary hCEC cultures were obtained from 24 research-grade corneas (mean donor age 73 (±8) years) not eligible for transplantation. Descemet membrane- endothelial cell (DM-EC) sheets were exposed to enzymatic collagenase digestion. Time to reach confluence was recorded for each culture and after passaging twice, cells were evaluated by immunohistochemistry.

Results: Pre-screening showed an enzymatic activity of 2.8 U/mg N-(3-[2-Furyl]acryloyl)-Leu-Gly-Pro-Ala (FALGPA) for the Sigma research-grade collagenase and 0.24 U/mg FALGPA for the Nordmark GMP-grade collagenase, while no significant differences in cell viability following human amniotic membrane digestion (used as a standard calibration method) were observed. Upon hCEC isolation, digestion of DM-EC sheets by the GMP-grade collagenase resulted in the formation of EC sheet fragments rather than hCEC clusters as observed upon DM-EC digestion by the research-grade collagenase. Primary hCEC cultures of cells isolated with the GMP-grade collagenase reached confluence faster than those of cells isolated with the research-grade collagenase (7 (±3) days vs 16 (±8) days, $P<0.05$). Primary hCEC derived from tissue digestion with either collagenase could be successfully passaged once, but after second passage cell cultures of cells isolated with GMP-grade collagenase either failed to reach confluence or displayed polymorphism and exhibited features of cellular stress.

Conclusion: The choice of collagenase can have a profound impact on the hCEC culture protocol for research grade corneas from elderly donors. While the GMP-grade collagenase tested in this study allowed to obtain confluence with high cell density within a week, upon further passage these cells exhibited increasing signs of cellular stress. In given conditions, our results render this

collagenase an unsuitable option for GMP-compliant cell culture protocols for clinical applications.

KEYWORDS: human corneal endothelial cells, cell culture, cell isolation, tissue engineering, GMP-compliant, collagenase.

INTRODUCTION

Cultured human corneal endothelial cells (hCEC) constitute the source for tissue-engineering approaches developed to overcome the global shortage of donor corneas. While cell injection therapy has already been tested in a clinical trial,[1] transplantation of bio-engineered cell-carrier constructs is still in the pre-clinical stage. Both approaches require reproducible cell culture protocols for primary hCEC and compliance with regulatory demands.

Establishing a reproducible protocol for the *in vitro* hCEC propagation is challenging, since the proliferative capacity of hCEC is influenced by many factors, including donor cell density. Low hCEC density at initiation of the culture can promote endothelial-to-mesenchymal transition and might have a general negative impact on morphology and proliferation *in vitro*.[2,3] Therefore, current studies preferably select young donor corneas and those with >2300 cell/mm^2, i.e. donor corneas that would be first line choices for transplantation.[4] Cultivated hCEC from older donors, on the other hand, have lower proliferation potential, and a lower threshold for endothelial-to-mesenchymal transition.[5] Elderly donors, however, constitute the majority of all donors (about 50% of all donors in 2018 in the United States were older than 60 years). Their corneal tissue can be used successfully for corneal transplantation and recently, attempts were presented to address the problem of initiating cell cultures from older donors with low cell density.[6,7] Small modifications to the cell culture protocol improved the success rate for these research-grade corneas and showed that the potential of these corneas has not yet been fully exploited. As cell isolation is one of the critical steps within a robust and reproducible protocol for endothelial cell expansion, in the current study we tested a further modification to the cell culture protocol to compare the effects of two different collagenases on hCEC isolation in order to improve the culture protocol for research-grade corneas from elderly donors. Starting from our previously established hCEC isolation and culture protocol, we compared the proliferation rate of primary cultures derived from hCEC isolated with a research-grade collagenase routinely used until now in our cell culture protocol and a GMP-compliant collagenase not described for being used for hCEC cell isolation until now.

MATERIALS AND METHODS

Materials

Collagenase NB 6 GMP grade from *Clostridium histolyticum* (Cat. No. N0002779, Lot No. 23540115) was obtained from Nordmark Arzneimittel GmbH & Co. KG (Uetersen, Germany) and Collagenase A from *Clostridium histolyticum* (Cat. No. C9722, Lot No. SLBM5274V) from Sigma-Aldrich Chemistry BV (Zwijndrecht, The Netherlands). Dulbecco's modified eagle medium (DMEM), L-ascorbic acid 2-phosphate, paraformaldehyde (PFA), ethylenediaminetetraacetic acid (EDTA), Triton X-100, 4',6-diamidino-2-phenylindole (DAPI), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Chemistry BV (Zwijndrecht, The Netherlands). Dulbecco's phosphate-buffered saline (PBS), fetal bovine serum (FBS), TrypLE™ Express, anti-ZO-1/TJP1 primary antibody (#617300), and secondary antibody were obtained from Life Technologies Europe BV (Bleiswijk, The Netherlands). Collagenase Activity Assay Kit was purchased from Abcam (Cambridge, United Kingdom). Pen/Strep Pre-Mix was purchased from Carl Roth GmbH + Co. KG (Karlsruhe, Germany). Fibronectin, collagen, and albumin (FNC) coating mix was purchased from Athena ESTM (Baltimore, MD, USA). Trypan Blue solution 0.04% (Hippocratech, Rotterdam, The Netherlands) was used to estimate hCEC vitality throughout the cell isolation and expansion protocol, and to allow the visibility of the Descemet Membrane-endothelial cell (DM-EC) sheets during preparation.

Pre-screening of collagenases

Pre-screening of the collagenases included comparison of the enzymatic activities of the research-grade collagenase ("Sigma collagenase") and the GMP-compliant collagenase ("Nordmark GMP grade collagenase") by a colorimetric assay (**Table 1**) with Collagenase Activity Assay Kit (Abcam, Cat. No. ab196999, UK). Collagenase stock solutions of 10 mg/mL were prepared with PBS (Sigma Aldrich D8662, USA), containing 0.133 $CaCl_2$ g/L. The collagenase stock solutions were diluted in DMEM (Sigma Aldrich, D1145, USA), containing 0.265 $CaCl_2$ g/L without phenol red resulting in final concentrations of 2

mg/mL or approximately 0.45 PZU/mL, and further diluted from there to suit the range of the assay (1:4.5, 1:9, 1:45). The samples were prepared according to the manufacturer's protocol of the Collagenase Activity Assay Kit. Briefly, samples diluted in assay buffer, positive control, provided by the assay kit, and negative controls (assay buffer) were pipetted into the wells of a 96-well plate (Greiner 96-well microplate, Cat.no. 655101, Austria) all adjusted to a volume of 100 µL/well. The collagenase reaction mix, containing 60 µL collagenase assay buffer and 40 µL bacterial collagenase substrate (N-(3-[2-Furyl]acryloyl)-Leu-Gly-Pro-Ala (FALGPA)), was added to each well and mixed. Measurement was performed in kinetic mode on a microplate reader (BMG Labtech, Polarstar Omega, Germany). Kinetic measurement was performed measuring every 2 min for 5 – 15 min at 37°C at OD345 nm. Two time points (T1 and T2) within the linear phase of the reaction progress curves were chosen to obtain corresponding OD values at these points (OD1 and OD2). Background control ΔOD value was subtracted from each sample value. Collagenase activity (U/mL) was calculated using the following formula: $((\Delta ODc/\Delta)\ 0.2\ x\ D/0.53\ x\ V)$ = FALGPA [U/mL].

For pre-screening of cellular viability after enzyme exposure, biopsies of human amniotic membrane (8 mm diameter, 6 biopsies) of 3 donors were obtained from Ludwig Boltzmann Institute (Vienna, Austria) and digested under constant shaking with 2 mg/mL Sigma collagenase or 1-4 mg/mL Nordmark GMP grade collagenase for 2 – 3.5 h at 37°C and humidified atmosphere. By this procedure human amniotic mesenchymal stromal cells (hAMSCs) were released from the tissue. Remaining tissue was further digested under constant shaking with 0.05 % trypsin (Sigma, T4174, USA) for 50 min at 37°C and humidified atmosphere, to release human amniotic epithelial cells (hAECs) from the tissue. Both cell populations hAMSCs and hAECS were stained with trypan blue (Merck, Cat. No. 11732, Germany) and viable and non-viable cells were counted in order to determine cellular viability.

Company	Collagenase	Origin	Cat. no.	Lot no.	GMP	PZ activity	FALGPA	FALGPA for 2 mg/mL
Sigma Aldrich	Collagenase A	*C. histolyticum*	C9722	SLBM5274 V	no	-	0.5 – 5.0 units/mg	1-10 U/ml
Nordmark	Collagenase NB 6	*C. histolyticum*	N0002779	23540115	yes	0.225 PZU/mg	-	~ 1.8 U/ml

Table 1: Specifications of Collagenases. Enzymatic activity unit definition: PZ activity (Wünsch) 1 PZ U/mg is estimated to be approximately 3.9 FALGPA U/mg; FALGPA One unit of Collagenase hydrolyzes 1.0 μmole of FALGPA (N-(3-[2-Furyl]acryloyl)-Leu-Gly-Pro-Ala) per minute at 25°C, at pH 7.5 in the presence of calcium ions.

Research-grade human corneoscleral tissues

24 research-grade human corneas not eligible for transplantation and obtained from 20 donors with a mean age of 73 (±8) years (range 58-85 years, **Table 2**) were included in the study. There was no difference in donor age (73 (±7) years vs 73 (±8) years, P=0.47) and in donor endothelial cell density (2258 (±240) cells/mm^2 vs 2117 (±339) cells/mm^2, P=0.06) between the two groups. All donor corneas were obtained from Amnitrans EyeBank Rotterdam and had an intact and viable endothelium. In all cases, donors had stated to have no objection against transplant-related research and the study was conducted according to the tenets of the Declaration of Helsinki and the Barcelona Principles.[8]

Isolation and growth of human corneal endothelial cells

Primary hCEC were isolated from DM-EC sheets using a two-step, peel-and-digest method as previously described.[9,10] After peeling, DM-EC sheets were stored in a growth factor-depleted medium for 4-7 days before hCEC isolation and expansion.[11] Upon tissue digestion, DM-EC sheets were exposed to two different enzymatic treatments: a 2 mg/ml Sigma collagenase (in DMEM) solution, and a 4 mg/ml Nordmark GMP grade collagenase (in DMEM) solution, both for 4 hours. Dislodgement of hCEC from DM was conducted at

37ºC and 5% CO_2 and hCEC clusters were further dissociated into single cells with TrypLE™ Express incubation for 5 minutes at 37ºC. The resulting cell population was plated onto organ-tissue plates previously coated with FNC coating mix and cell density was determined by using 10 µl of cell suspension for an automatic cell count (Spark™ 10M multimode microplate reader, Tecan Trading AG, Männedorf, Switzerland). When reaching 80% to 90% confluence, the culture medium was replaced with stabilization medium for the next 2-4 days before passaging, to enhance the cellular morphology.[12] Primary cultures were then passaged using TrypLE™ Express and sub-cultured at a 1:2 splitting ratio on FNC-coated culture well plates. The morphology of cultured hCEC was assessed throughout the whole culture period with an AxioVert.A1 microscope with AxioCam ERc 5s stand-alone functionality camera (Zeiss, Oberkochen, Germany).

Donor information	Indicators
Number of corneas (donors)	24 (20)
Gender, n	
Male	11
Female	9
Mean age (±SD), yrs (range)	73 (±8), (58-85)
Mean storage time* (±SD), days (range)	14 (±6), (7-25)
Mean ECD (±SD), cells/mm^2 (range)	2188 (±302), (1800-2800)
Cause of death, n	
Respiratory	9
Cardio/Stroke	5
Other	3
Malignant	3

Table 2: Demographics of donor data. *Mean storage time = time between death and culture of first isolated DM-EC tissue; SD = standard deviation; yrs = years.

Immunofluorescence

For hCEC characterization after second passage the expression of the structural marker ZO-1 was analyzed.[13] Briefly, hCEC were fixed with 4% paraformaldehyde in PBS or methanol for 15 minutes at room temperature. Next, samples were permeabilized using 0.1% Triton X-100 and incubated with 3% BSA-based blocking buffer in PBS for 30 minutes. Samples were subsequently incubated with primary antibodies anti-ZO-1 tight junction protein (anti-ZO-1/TJP1, dilution 1:100) for 1 hour, followed by washing steps with PBS and incubation with a secondary antibody (dilution 1:200) in the dark for 45 minutes. Further, the samples were stained with the DNA-specific dye DAPI, and then imaged using an inverted fluorescence microscope.

RESULTS

Colorimetric Analysis of Collagenase Activity and Cellular Viability

Collagenase activity was determined to be 5.6 U/ml FALGPA for Sigma collagenase and 0.48 U/ml FALGPA for Nordmark GMP grade collagenase solutions of the same weight per volume concentration. Accordingly, for 2 mg/mL collagenase solutions, the enzyme activity was determined to be 2.8 U/mg FALGPA for Sigma collagenase and 0.24 U/mg FALGPA for Nordmark GMP grade collagenase (**Figure 1, Table 3**).

To ensure, that the given enzyme concentration had no negative impact during isolation of human cells, human amniotic membrane was degraded for extended times (2-3.5h). Enzymatic tissue degradation of human amniotic membrane with Sigma collagenase or Nordmark GMP grade collagenase with the given activity range had no impact on viability of isolated hAMSCs and hAECs (95.1-100 %).

Digestion of DM-EC sheets and *in vitro* endothelial cell expansion

Based on the colorimetric analysis results which indicated different enzymatic activities for both collagenases (**Table 3**), the Nordmark GMP-grade collagenase was first tested for different concentrations in the range of 1-4 mg/mL and for different digestion times. A concentration of 2 mg/ml for the Nordmark GMP-grade collagenase resulted in very large DM-EC fragments even after digestion times of >4 hours. Raising the collagenase concentration to 4 mg/ml and using digestion times of 4 hours resulted in smaller fragments that were appropriately sized in order to avoid longer trypsin exposure times later in the protocol which might be potentially harmful for the cells.

Enzymatic digestion of the DM-EC sheet by the Sigma collagenase for 4 hours resulted in a total digestion of the DM (**Figure 2A**) and a subsequent flushing step was applied to allow for a homogeneous dislodgement of large hCEC clusters into smaller-sized clusters (**Figure 2B**). In contrast, after enzymatic digestion of the Nordmark GMP grade collagenase exhibited some of the DM to be still attached to the cell sheet after 4 hours of digestion (**Figure 2C**) and

in the following flushing step, the endothelial cell layer fragments were further dislodged from the DM into smaller hCEC clusters (**Figure 2D**).

Cell counting performed on the cell suspensions before seeding showed a higher cell density for the suspensions after digestion by the Nordmark GMP grade collagenase (average seeding density of 18608 ± 10230 cells/ml of cell suspension) compared to the cell density after Sigma collagenase-mediated tissue digestion (average seeding density of 4759 ± 3185 cells/ml of cell suspension) (P=0.04).

During *in vitro* cell expansion, a significantly faster proliferation rate (time to reach cell confluence at P0) was observed in all cultures obtained from DM-EC sheets digested with Nordmark GMP grade collagenase (7 ± 3 days) compared to hCEC cultures established after DM-EC sheet digestion with Sigma collagenase (16 ± 8 days, P=0.03). Cells in both types of culture retained the typical cobblestone shape of endothelial cells (**Figure 3 A,B**). After first passage no variations in proliferation time was observed, regardless the enzyme used from DM-EC sheets digestion (**Figure 3 C,D**). After second passage, however, morphology of isolated cells with the GMP-grade collagenase showed a high degree of variability.

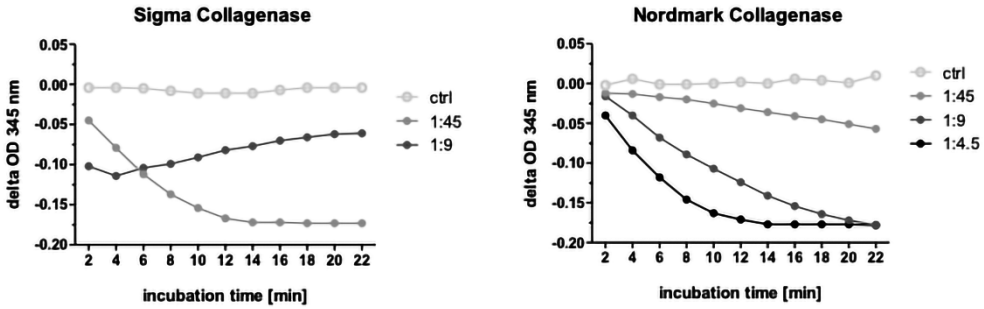

Figure 1: Kinetics of collagenase activities. Kinetics of enzymatic reaction of Sigma Collagenase and Nordmark Collagenase NB 6 GMP grade, measured with Collagenase Activity Assay Kit. OD was measured at 345 nm, and time intervals of measurement were 2 min. Ctrl = negative control. 1:4.5, 1:9, 1:45 = dilutions of the respective collagenase.

Cells appeared larger in size, exhibited signs of cellular stress or were unable to reach confluence at all (**Figure 4 A-C**) as compared to research-grade collagenase-derived cultures (**Figure 4D**). Presence of cellular stress was also reflected by a fragmented expression of ZO-1 at cell borders throughout the layer or showing non-contact inhibited cells (**Figure 5 A,B**).

Collagenase	Collagenase solution (mg/ml)	FALGPA (U/ml)	FALGPA (U/mg)
Research-grade collagenase (Sigma Aldrich)	2	5.60	2.8
GMP-compliant collagenase (Nordmark)	2	0.48	0.24

Table 3. Calculation of Collagenase Activity. Calculations were performed as follows: $((\Delta ODc/\Delta)\ 0.2\ x\ D/0.53\ x\ V)$ = FALGPA [U/mL] (ΔODc = OD2 – OD1 = ΔOD reading from sample at T2 and T1, corrected for background; ΔT = linear phase reaction time T2 – T1 (minutes). 0.2 = reaction volume (mL); D = sample dilution factor; V = sample volume added into the reaction well (mL); 0.53 = millimolar extinction coefficient of FALGPA.

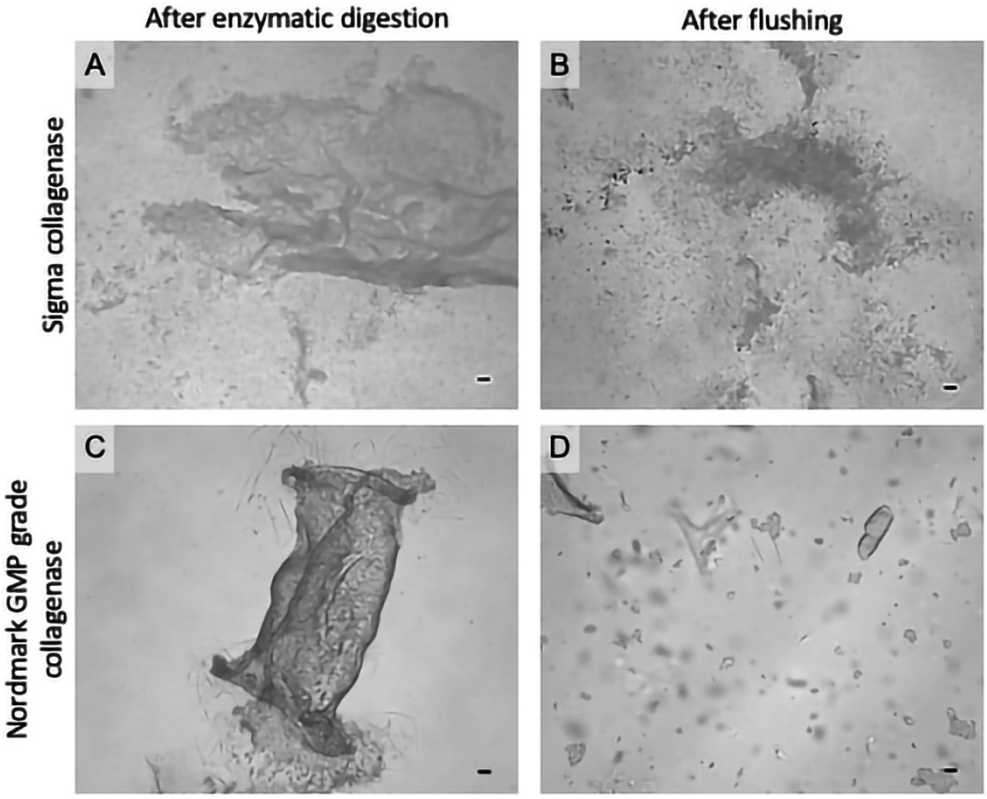

Figure 2: Enzymatic digestion of DM-EC sheets. Series of images illustrating the different outcomes of DM-EC sheet digestion when using Sigma collagenase (A,B) and Nordmark GMP-compliant collagenase (C,D), respectively. Scale bars = 100 μm.

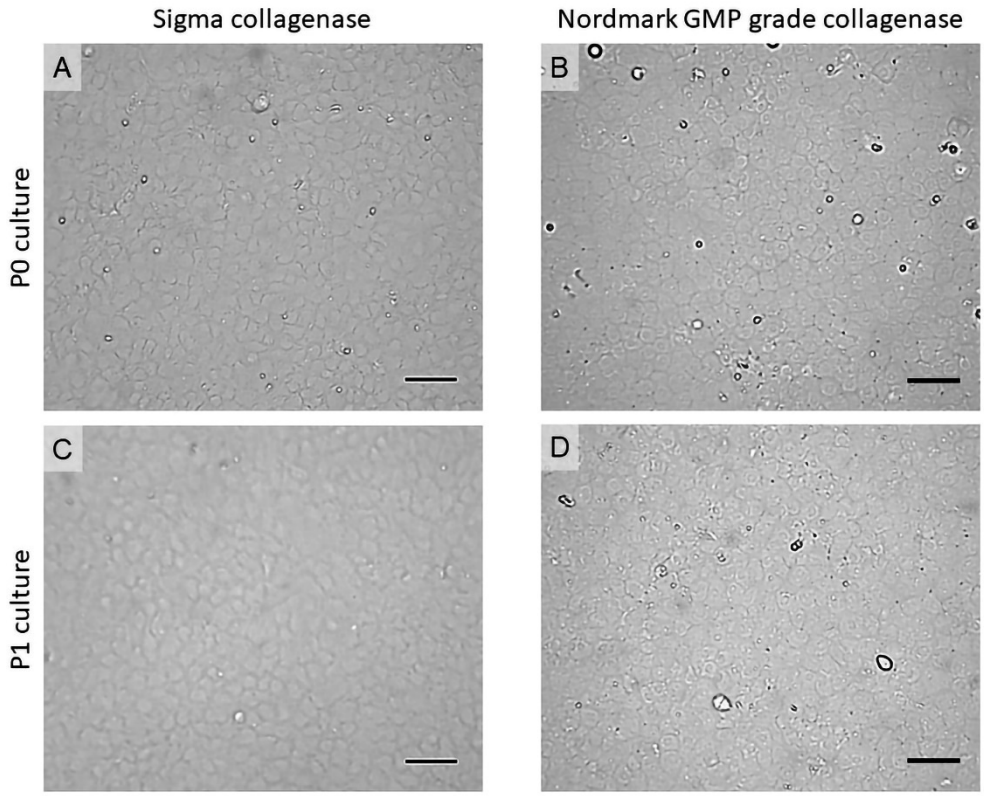

Figure 3: Cultured hCEC isolated from different digestion methods. Light microscopy images representing confluent hCEC cultures from P0 to P1 and isolated from DM-EC sheets following digestion with Sigma collagenase (A,C) and Nordmark GMP-compliant collagenase (B,D), respectively. Scale bars = 100 μm.

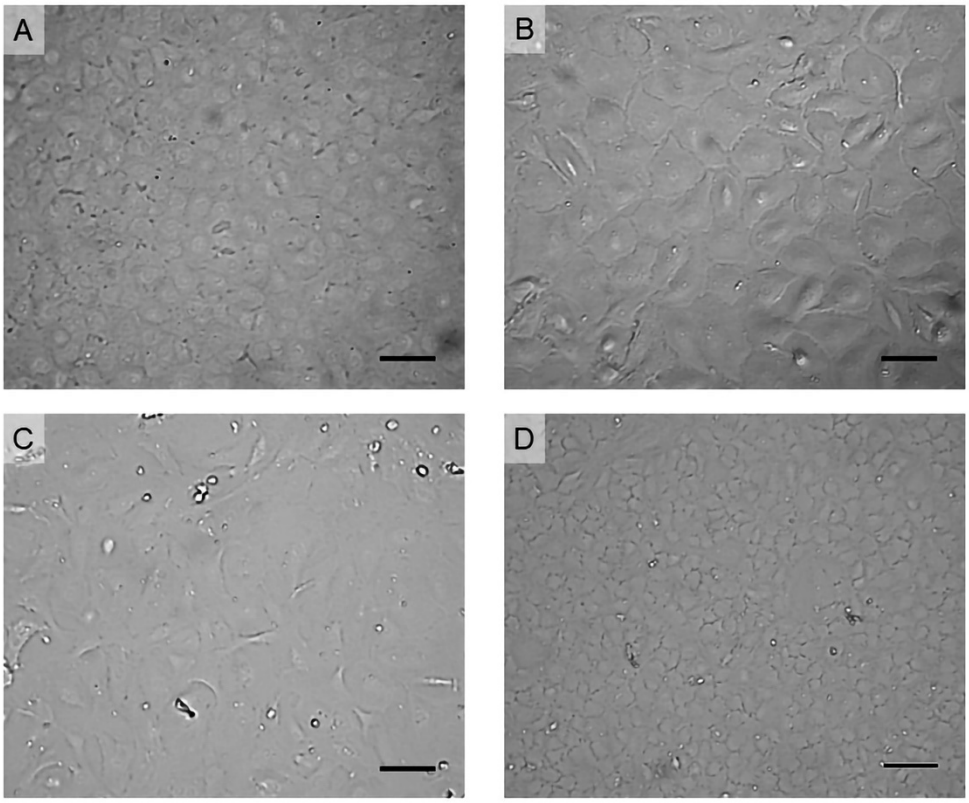

Figure 4: Cultured hCEC at P2. Series of images showing the differences present in hCEC cultures at P2. Cells expanded after isolation with Nordmark GMP-compliant collagenase looked stressed (A), larger in size (B) or were not able to reach cell confluence and good endothelial morphology (C). On the other hand, cultures established following isolation with Sigma collagenase reached a good degree of cell confluence and cells looked smaller (D). Scale bars = 100 μm.

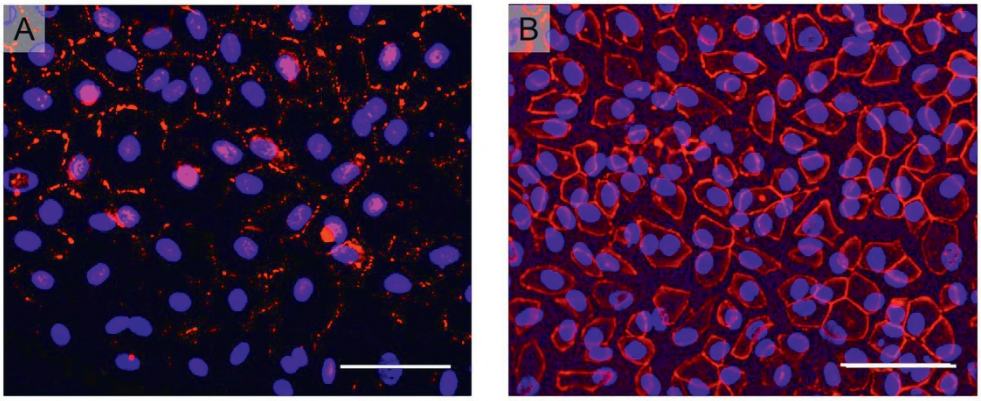

Figure 5: ZO-1 expression on P2 cultures. Immunofluorescence images displaying the expression pattern of the tight junction protein ZO-1 on P2 cultures expanded after tissue digestion with Nordmark GMP-compliant collagenase. Because of the nature of the cells, expression resulted fragmented (A) or highlighted the non-contact inhibition between cells (B). Scale bars = 100 μm.

DISCUSSION

In this study, we analyzed the enzymatic properties of a GMP-grade collagenase and its effect on a previously established *in vitro* hCEC isolation and culture protocol.

The isolation of intact cells from complex tissues still represents a considerable challenge.[14] For corneal endothelial cells, different types of collagenase have been employed for cell isolation, either research-grade or GMP-grade collagenases.[3,12,15,16] Implementation of a GMP-grade collagenase for the cell isolation step is mandatory of compliance with regulatory demands.[17] The GMP-grade collagenase employed in this study had already been successfully applied for isolation of human pancreatic islets,[18] but not for isolation of hCEC. Enzymatic activity of this collagenase proved to be lower than for the research-grade collagenase routinely used in our culture protocol[11] and an increase in its concentration was required for effective DM digestion. Despite the higher concentration, the GMP-grade collagenase appeared to exert a less disruptive effect on the intercellular hCEC connections which in turn resulted in a higher cellular yield. For DM-EC sheets digested with the GMP-grade collagenase, an increase of cellular yield of about 4 times was observed. This is in contrast to results described by Peh et al., who reported no difference in cellular yield when comparing digestion by a GMP-grade collagenase blend (Liberase TH) and a research-grade collagenase (Collagenase Type 1).[19] This might be explained by the superior quality of their research-grade corneas (median donor age of 22 years vs 73 years in this study), resulting in an higher overall cellular yield or by an initial higher yield obtained with Collagenase I compared to Collagenase A.

The higher cell concentration in the seeding cell suspension obtained after the DM-EC sheet digestion with GMP-grade collagenase led on average to shorter times to reach a confluent, uniform cell layer, but with no difference in cell morphology at confluence in cultures derived from the GMP-grade and the research-grade collagenase isolation, respectively. After the first passage, no difference in the time to reach confluence was observed between cultures derived by the two types of collagenases. However, despite the initial high cell density and hexagonal morphology at P0, at second passage cells isolated with the GMP-grade collagenase showed variable outcomes. While some cultures

failed to reach confluence, in other cultures cells exhibited signs of cellular stress. This may indicate that the initial cell isolation process under the conditions applied in this study may induce more cellular stress as compared to the research-grade collagenase, which upon passaging results in a decrease in proliferative capacity. On the other hand, an assay for testing the impact of the applied enzyme concentration on human cells was conducted before starting out the cell isolation experiments and this assay showed no adverse effects of the applied collagenase concentration on amniotic tissue. This would imply that either the assay was not sensitive enough to extrapolate the outcome to human corneal endothelial cells or the cellular stress is induced after the cell isolation step leading to a transient growth arrest in which endogenous telomerase enzymes needed for telomeric overhang from erosion were probably lost in these cells.[20]

It should be pointed out, however, that the higher cellular yield obtained upon tissue digestion with the GMP-grade Nordmark collagenase within this study, had a beneficial effect on starting hCEC cultures derived from elderly donors. Endothelial cells from elderly donors are generally considered as challenging for establishing successful cell cultures. It has been shown earlier that the age of cornea donors represents a critical parameter in the establishment of a reliable and reproducible endothelial cell culture protocol, as these cells have a lower proliferation capacity.[21] However, as the majority of donor corneas derives from elderly donors, these donors constitute a valuable pool of research-grade corneas for transplant-related cell culture studies. In previous studies, modifications to existing cell culture protocols have been suggested to improve the success rate of establishing cell cultures from elderly donors. Parekh et al. reported that forced cell attachment by using hyaluronic acid and supplementing the culture medium with ROCK inhibitor Y-27632 had a beneficial effect on the culture of endothelial cells isolated from old donor corneas.[7] In addition, we described the beneficial use of a modified dual-media approach for establishing confluent hCEC cultures from elderly donors. In that study, research-grade collagenase A was employed for DM-EC sheet digestion.[11] However, a replacement of a research-grade component by a GMP-compliant alternative is essential for fulfilling regulatory demands for potential clinical applications. Since in the current study, tissue digestion by the Nordmark GMP grade collagenase resulted in higher cellular yield than the research-grade collagenase A digestion,

implementing the Nordmark GMP grade collagenase in the cell isolation protocol could have been considered as another step towards establishing a robust cell culture from elderly donor corneas for potential clinical applications. Currently, however, additional studies on the dependency of induced cellular stress on the applied collagenase concentration and how stress induction may be mitigated by additional modifications in the culture protocol may therefore be required before incorporating this tested GMP-grade collagenase into culture protocols.

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