Characterization of cryopreserved primary human corneal endothelial cells cultured in human serum supplemented media

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INTRODUCTION

Since Stocker et al.[1] established human corneal endothelial cell (HCEC) culture, the potential for cell therapy for corneal endothelial dysfunction using HCECs has shown continuous evolution. [2] The limitations associated with this therapy can basically be divided into two major areas: those related to culturing the cells, such as proliferation, cellular senescence, and fibroblastic transformation; and those related to the logistics and techniques for transplanting the cells.[2]

Regarding the first issue, we have recently published a paper showing the use of human serum supplemented media (HS-SM) instead of fetal bovine serum supplemented media (FBS-SM), suggesting some potential advantages of a media with fewer animal derived contents.[3] Regarding the second issue, cryopreservation of corneas for transplant[4] and of cultured HCEC using standard medium (FBS-SM) have already been shown[5] and could potentially improve the logistics of distributing cultured HCECs for clinical use. The purpose of the present study is to determine the ability of HCECs grown in HS-SM to be cryopreserved and maintain their characteristics by comparing them to cryopreserved cells grown in FBS-SM.

MATERIALS AND METHODS

Three pairs of human corneas from donors aged 8, 28, and 31 years old were obtained from Lions VisionGift (Portland, OR) and the Alabama Eye Bank (Birmingham, AL). From each pair, one cornea was used to start a HCEC culture with HS-SM and the other cornea with FBS-SM. Upon reaching confluence, the 6 cell populations were frozen using 10% dimethyl sulfoxide containing medium (resumed methodology shown in **figure 1**). Thawed cells grown in HS-SM were compared to FBS-SM by morphology (**figure 2**), growth curves (**figure 3**), immunohistochemistry (**figure 4**), real time-reverse transcriptase polymerase chain reaction (RT-PCR) for endothelial cell markers (**figure 5**), and detachment time (**figure 6**).

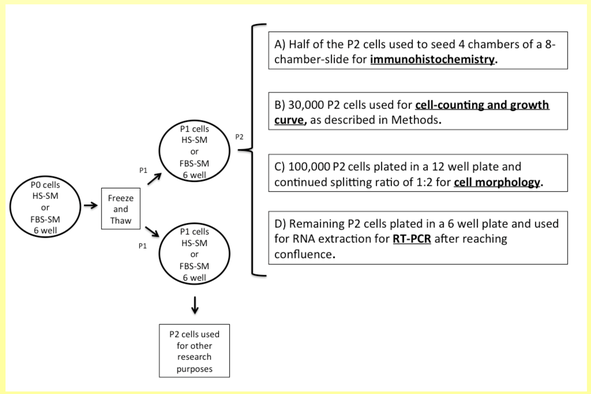

Figure 1. Methodology of HCEC passages and their use. P: passage; FBS-SM: fetal bovine serum supplemented media; HS-SM: human serum supplemented media; RT-PCR: real time-polymerase chain reaction.

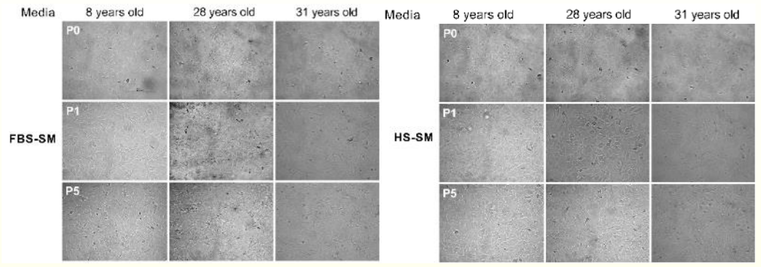

Figure 2. Phase-contrast images of confluent passage 0 (before cryopreservation) and passages 1 and 5 (after cryopreservation) HCECs cultured in fetal bovine serum supplemented media (FBS-SM) and human serum supplemented media (HS-SM). Similar morphology is seen between the two media for the same cell population and passage. All pictures were taken using 20x magnification. Scale bar = 200 micrometers.

Figure 3. Ten day growth curve comparing cells cultured in fetal bovine serum supplemented media and human serum supplemented media starting at passage 2. Day 1, 2, 3, 5, and 7 counts were obtained for cells seeded in a 24-well plate, day 10 counts for cells seeded in a 6-well plate. Error bars represent standard deviations.

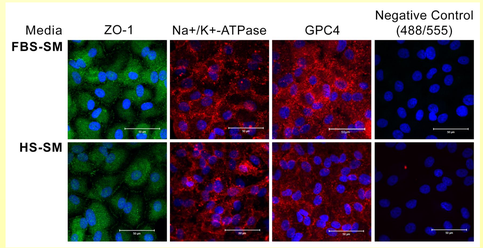

Figure 4. Immunostaining for zonula occludens-1 (ZO-1), sodium-potassium ATPase (Na+/K+-ATPase) and glypican 4 (GPC4) of confluent passage 2 HCECs cultured in media with fetal bovine serum (FBS-SM) and human serum (HS-SM). Staining for ZO-1 is from the 31 year old donor, Na+/K+-ATPase and Negative Control (secondary antibodies AlexaFluor 488 (above) and 555 (below) only) from the 8 year old donor, and GPC4 from the 31 year old donor. Scale bars = 50 micrometers.

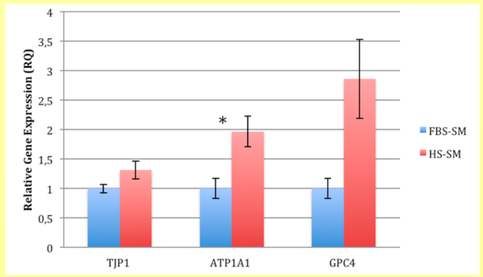

Figure 5. RT-PCR of tight junction protein 1 (TJP1); ATPase, Na+/K+ transporting, alpha 1 polypeptide (ATP1A1); and glypican 4 (GPC4) of passage 2 HCECs cultured in media with fetal bovine serum (FBS-SM) and human serum (HS-SM). FBS-SM is presented as the reference (RQ=1). * $p<0.05$.

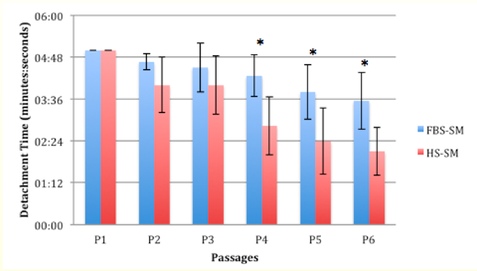

Figure 6. Average detachment time of all cell populations from P1 to P6, comparing cells cultured in FBS-SM and HS-SM. Maximum time allowed to detach was 5 minutes. Error bars represent standard deviations. * $p<0.05$.

RESULTS

No difference in morphology could be seen for cells grown in the two media before or after cryopreservation. By growth curves, cell counts after thawing were similar in both media, with a slight trend toward higher cell counts in FBS-SM. Cells grown in both media showed similar expression of endothelial cell markers when assessed by immunohistochemistry, although the gene expression of HCEC markers was higher in HS-SM when assessed by RT-PCR. There was a tendency of longer detachment time with FBS-SM and lower passages.

CONCLUSIONS

HS-SM was similar to FBS-SM for cryopreservation of cultured HCECs when assessed by cell morphology, proliferation, and protein expression, although marker gene expression by RT-PCR was higher in HS-SM compared to cells grown in FBS-SM. Detachment time was longer with FBS-SM and in lower passages. .

REFERENCES

1) Stocker FW, Eiring A, Georgiade R, et al. A tissue culture technique for growing corneal epithelial, stromal, and endothelial tissues separately. Am J Ophthalmol. 1958;46(5 Part 2):294-8. 2) Okumura N, Kinoshita S, Koizumi N. Cell-based approach for treatment of corneal endothelial dysfunction. Cornea. 2014;33 Suppl 11:S37-41. 3) Vianna LM, Kallay L, Toyono T, et al. Use of human serum for human corneal endothelial cell culture. Br J Ophthalmol. 2014;0:1-5.4) Brunette I, Le François M, Tremblay MC, et al. Corneal transplant tolerance of cryopreservation. Cornea. 2001;20(6):590-6. 5) Suh LH, Zhang C, Chuck RS, et al. Cryopreservation and lentiviral-mediated genetic modification of human primary cultured corneal endothelial cells. Invest Ophthalmol Vis Sci. 2007;48(7):3056-61.

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DISCLOSURES

The authors declare no commercial relationships relevant to this work.