Human corneal endothelial cell assessment from the tissues preserved in serum-based and synthetic storage media

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Running title:
Corneal endothelial cell assessment

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Abstract:

Aim: To assess the difference between endothelial cells from the tissues preserved in media supplemented with fetal bovine serum (FBS) and recombinant human serum albumin (rHSA).

Methods: In a donor matched study, 48 tissues were preserved for 28 days at 31°C in Cornea Max® and Cornea Syn® supplemented with FBS and rHSA respectively. Endothelial cells were visualized by two masked observers before and after preservation. Endothelial cell density (ECD) and number of iatrogenic folds were counted manually. Alizarin red staining and tight junction protein (ZO-1) were used to assess cell morphology (hexagonality and polymorphism). Intra and inter-observer cell counts were recorded and analyzed. Wilcoxon and one-way ANOVA tests were used where p<0.05 was deemed statistically significantly different.

Results: Significant amount of iatrogenic folds were observed in the tissues supplemented with FBS compared with rHSA post-preservation (p=0.0007). Approximately 69% and 71% hexagonal cells (p=0.0303) and; 29% and 26% polymorphic cells (p=0.0234) were observed in FBS and rHSA groups, respectively. Post-preservation, operator 1 counted 1766 cells/mm² in FBS and 1864 cells/mm² in rHSA. Operator 2 counted 1702 cells/mm² in FBS and 1858 cells/mm² in rHSA. ECD counts from FBS (inter-operator) were statistically significant (p=0.0429). However, significance was not observed in the ECD counts (inter-operator) from the rHSA preserved tissues (p=0.8738).

Conclusions: rHSA supplemented media allows better visualization of the corneal endothelial cells. This reduces the rate of discard observed due to counting errors. Use of rHSA improves the current standard of care and reduces the use of animal derived products.
Keywords:

Eye bank; cornea; preservation, organ culture; synthetic medium; recombinant human serum albumin
Introduction:

Human corneas are harvested from their cadaveric donors with full consent from the donor’s next-of-kin to be used for transplantation or research. Eye banks are responsible for collecting, processing, monitoring, evaluating and shipping the tissues for surgical use with strict selection criteria\(^1\). One of the main parameters include endothelial cell assessment. According to the European eye bank association guidelines, corneal tissues with <2,000-2,200 endothelial cells/mm\(^2\) cannot be used for corneal transplant\(^2\).

Endothelial cell density (ECD) is one of the most essential parameters that differentiate a transplantable grade tissue from a research quality tissue. However, those grafts that are on the borderline (2,000-2,200 cells/mm\(^2\)) must be thoroughly evaluated to reduce the number of discarded tissues due to incorrect counting measures. Precise pre-surgical endothelial cell evaluation is also important as this may directly affect the post-surgical endothelial cell loss measurements\(^3\)\(^-\)\(^5\).

Organ culture (OC) preservation of corneas is widely used in Europe. During OC, significant amounts of deep iatrogenic folds are generated. There is often a difference in endothelial cell counts that is noted before and after preservation due to the change in its physiological state. Endothelial cell counts can be performed by swelling the intercellular borders, that make the endothelial cells easily visible when viewed under a microscope. At our institute, we use 1.8% sucrose solution for dilating the intercellular borders by means of osmosis. The number of cells were then counted using a calibrated reticule\(^6\)\(^-\)\(^8\). However, in the United States, the eye banks rely on a specular microscope and hypothermic storage method to preserve the donated corneas. Regardless of the method used for counting, visualization of clear cell borders can improve the precision while counting the number of endothelial cells.
Inter and intra-bank variability has previously been reported\(^9\). Perfectly aligned cell membrane visualization with alizarin red staining has shown a precision range between +5 and -5\(^\%\)\(^\text{10}\). If the tissues have iatrogenic folds, it increases the difficulty level of cell counts resulting in a significant counting error. The precision, however, is still dependent on the observer. In our earlier report, we showed that tissues preserved in recombinant human serum albumin (rHSA) have better endothelial cell viability and overall tissue quality\(^\text{11}\). This study aims at comparing the endothelial cell visualization of the tissues preserved in the media supplemented with fetal bovine serum (FBS) i.e. Cornea Max\(^\circledR\) (Eurobio, Les Ulis, France) and rHSA i.e. Cornea Syn\(^\circledR\) (Eurobio, Les Ulis, France).
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Methods:

Ethical statement

The tissues were obtained by Fondazione Banca degli Occhi del Veneto Onlus, Venice, Italy with full consent from the donor’s next-of-kin to be used for research. ECD of the tissues was <2,200 cells/mm² without any other co-morbidities. One corneal tissue from the same donor was preserved in FBS supplemented media and the contralateral tissue was preserved in rHSA supplemented media for 28 days at 31°C.

Tissue evaluation

Morphology (n=48) of the tissues was visualized using Trypan blue stain (0.25% wt/vol) (VisionBlue, D.O.R.C., Zuidland, The Netherlands) to evaluate the percentage of dead/necrotic cells before and after preservation. The endothelium was exposed to a hypotonic sucrose solution, which helps counting the number of endothelial cells and to examine the general morphology (pleomorphism and polymegathism). ECD was counted using a 10X10 reticule (0.1mm²) attached to the eyepiece of an inverted microscope (Axiovert, Zeiss, Germany) at 100x magnification. ECD was expressed as an average of five different counts, each performed at a different endothelial area. Number of folds were counted manually using the same set of images.

Alizarin red staining

Endothelium of the corneas (n=4, for each condition) was exposed to alizarin red stain for 3–5 minutes and washed with 1x PBS to check the morphology of the endothelial cells. The cells were observed using an inverted light microscope (Axiovert) and images were obtained using zen software (Zeiss, Milan, Italy).
Immunostaining for tight junctions using zonula occludens-1 (ZO-1)

The tissues (n=6, from each condition), after preservation, were washed with PBS and the Descemet membrane-endothelial complex was peeled using a standard stripping technique used for Descemet membrane endothelial keratoplasty (DMEK). The stripped DMEK tissue was fixed in 4% paraformaldehyde at room temperature (RT) for 20 minutes’. The cells were permeabilized with 0.5% triton x-100 for 30 minutes. After blocking with 5% goat serum for 1 hour at RT, the cells were incubated with primary antibody [anti-ZO-1 (pre-conjugated with FITC), 1:200 (ZO1-1A12, Thermo Fisher Scientific, Rochester, NY, USA)] for 3 hours at RT. After each step, the cells were sequentially washed three times with PBS. The stripped tissues were flat mounted, covered with the mounting medium (Vectorshield, Vector labs, CA, USA) and examined under a Nikon Eclipse Ti-E (Nikon, Burgerweeshuispad, Amsterdam) microscope using the NIS elements software (Nikon).

Measurements and statistical analysis

ImageJ (FIJI) software was used to measure and analyze the data. For ZO-1, the area was selected and a pre-defined command (macros) was used that converts the image to overlay masks. Total number of cells were automatically calculated by the software. Whereas, the hexagonal (all 6 borders in each cell) and polymorphic cells were counted manually based on the cell structure in a particular area.

Non-parametric Wilcoxon test for paired data and one-way ANOVA test for independent measures were used to check the statistical significance between FBS and rHSA preserved tissues, where p<0.05 was deemed statistically significant. A post-hoc correction to the significance was applied using Bonferroni test.
**Results:**

**General morphology**

The average number of folds counted before preservation in FBS (1.6±1.2) (Figure 1A) and rHSA (1.7±1.1) (Figure 1B) did not show any statistical difference (p=0.8114). The number of folds significantly increased (p=0.0007) after preservation of the tissues in FBS (4.3±1.5) (Figure 1C) compared with the tissues preserved in rHSA (2.9±0.8) (Figure 1D). At a higher magnification, counting the cells was slightly difficult due to increased thickness and amount of folds observed from the tissues preserved in FBS (Figure 1E) compared with those in rHSA (Figure 1F). The cells present on the folds were easily viewed without changing the magnification from the tissues preserved in rHSA group.

**Alizarin red staining**

Alizarin red staining showed several areas with deep folds from the tissues preserved in FBS group (Figure 2A) compared with rHSA group (Figure 2B), that did not show significant amount of folds. At multiple sites, large denuded areas were observed with significant changes in endothelial cell morphology on the tissues preserved in FBS (Figure 2C). rHSA preserved tissues did not show large denuded areas (Figure 2D).

**ZO-1 immunostaining, hexagonality and polymorphism analysis**

ZO-1 staining showed stretched and polymorphic cells (marked with white dashes) on the tissues preserved in FBS group (Figure 2E). This was not observed from the tissues preserved in rHSA group (Figure 2F). 68.71(±3.82)% hexagonal cells were recorded from the tissues preserved in FBS compared with 70.88(±2.80)% hexagonal cells from the rHSA group, which was found to be significantly different (p=0.0303). However,
the amount of polymorphic cells found in the FBS group was 28.79(±3.83)% compared
with 26.5(±2.84)% in the rHSA group, which was significantly different (p=0.0234).
This decreased number of hexagonal cells from the tissues preserved in the FBS group
is assumed to be because of cell stretching due to the generation of folds during the
preservation phase.

Inter and intra-operator variability
ECD counts before preservation from operator 1 between FBS (1885±156 cells/mm²)
and rHSA (1890±159 cells/mm²) (p=0.9443) and from operator 2 between FBS
(1895±167 cells/mm²) and rHSA (1900±163 cells/mm²) (p=0.9468) did not show any
statistical significance. No difference was found between the cell counts from operator
1 and 2 when the tissues were preserved in FBS (p=0.8918) and rHSA (p=0.8914).
ECD values after preservation between FBS (1766±112 cells/mm²) and rHSA
(1864±132 cells/mm²) from operator 1 was found to be significantly different
(p=0.0084). ECD values from operator 2 between FBS (1702±101 cells/mm²) and
rHSA (1858±138 cells/mm²) was found to be statistically significantly different
(p<0.001). However, the ECD values observed by two operators from the tissues
preserved in FBS was found to be significantly different (p=0.0429), but it did not show
any significance from the rHSA group (p=0.8738).
Pre- and post-preservation ECD counts from operator 1 on the tissues preserved in FBS
(p=0.0796) and rHSA (p=0.6594) did not show statistical significance. However, ECD
count between pre- and post-preservation from operator 2 on tissues preserved in FBS
(p=0.0084) was found to be significantly different in contrast to rHSA (p=0.5221),
which did not show statistical significance.
Discussion

Data from the European eye banks suggest that approximately 15-20% of the corneas are discarded due to poor endothelial cell counts (usually <2,000 cells/mm²). Hence, the tissues with borderline cell counts need to be precisely evaluated to primarily categorize it for transplantation or research purpose. This may have a huge impact on increasing or decreasing the number of corneal tissues suitable for transplant. It is important to note that ECD measurement before the tissue is shipped from the eye bank is one of the main parameters of corneal quality control, and therefore needs to be recorded precisely. In a study carried out by French eye banks, more than half of the cell counts showed deviations by more than 10% from their actual counts. The counts were over-estimated by 33% and under-estimated by 26%. This data relates to delivery of poor quality corneas for transplantation purposes in certain centers and an increased discard rate in others. Even computer-aided methods have failed to provide reliable results, mainly due to poor imaging of the cells.

In this masked, donor-matched study, we observed that visualization of the endothelial cells after preservation of the tissues in rHSA supplemented media was better than those preserved in FBS containing media. This further minimized the endothelial cell counting error. We do not claim that our cell counts were 100% reliable, as this depends mostly on the masked observer and can be subjective. However, as visualization of the endothelial cells from the tissues preserved in rHSA group was better, it reduced the risk of over- or under-estimation of cell counts. It was observed that the cells around the deep folds changed their morphology by stretching, which created difficulties in counting the cells at 100X magnification. An increase in the number of folds and cell stretching escalated the magnitude of cell counting errors, which was not observed in the tissues preserved in rHSA.
Corneal endothelial cell assessment

Cornea Max® is a routinely used OC media that contains FBS, which possibly creates haze due to turbidity, thus affecting the visualization and image quality of the endothelial cells. Cornea Syn® is supplemented with rHSA and could reduce this error significantly, as it allows clear observation and evaluation of endothelial cells. Importantly, better intercellular border visualization helps in reducing the cell counting errors and the overall time required for counting the number of cells, which plays a significant role in the field of eye banking, especially for high volume eye banks. As the cells from FBS could not be evaluated precisely due to the increased number of iatrogenic folds and poor cell visualization, the recorded ECD values were not as high as the tissues from the same donor that was preserved in rHSA post-preservation, thus increasing the chances of over- or under-estimation.

A gradual change of ionic content between the cells and the intercellular junctions may increase water egress from the cells, thus promoting dilation of the intercellular spaces in the presence of a hypotonic solution\textsuperscript{2}. The endothelial cell visualization after osmotic dilation of the intracellular spaces is usually affected after 2 weeks. However, we observed that the cell visualization remained constant even during the fourth week if the tissues were preserved in rHSA. This visualization improved from day 0 to week 1 of the storage and remained relatively constant thereafter up to week 4. However, slight deterioration in the cell visualization was observed with the FBS group during this time because of increasing thickness and generation of folds due to swelling. Endothelial cells from the tissues preserved in FBS were difficult to differentiate, and this may explain a difference in cell counts arising between the two operators. The final quality further improves the precision of cell counts and reduces the overall wastage of tissues deemed for transplantation due to improper endothelial cell counts\textsuperscript{6-8,10,16,17}.

Preserving corneal tissue in Cornea Syn® supplemented with rHSA has shown the
following advantages: a) It is fully synthetic and therefore complete deduction of animal or animal derived products is possible; b) It does not require additional batch-to-batch testing for maintaining good standards of practice; c) There is good visualization of the endothelial cells leading to ease of cell counting and reducing the under- or over-estimation, which has a huge impact on the tissue selection procedure; d) It minimizes the extended time period for counting the endothelial cells, and limits the loss of good quality tissues for transplantation; e) It improves the possibility of counting the cells at iatrogenic folds thus reducing the cell count errors due to deep folds; f) It maintains cell viability and; g) as the pre-endothelial cell counts are precise, it allows a more accurate evaluation of post-operative endothelial cell loss.

It is known that the preservation media is mostly selected based on economic arguments. As Cornea Syn® contains a recombinant ingredient, the overall cost could be relatively higher than the currently used Cornea Max® media. However, as the European laws favor the 3 ‘Rs’ policy and the advantages of using rHSA are greater, the benefits would compensate the economic cost. We have tested several parameters that are usually required for an eye bank to justify a tissue for transplantation, and have found that the rHSA series was effective in statistically compared parameters. The rHSA series therefore may constitute a substantial advancement in the tissue preservation and quality control assessment including ECD measurements.
References:


**Figure legends:**

**Figure 1:** Corneal endothelial cells as observed before preservation in A) FBS and B) rHSA supplemented media. Endothelial cells from the same tissues observed after preservation at 31°C in C) FBS and D) rHSA. At a higher magnification, the endothelial cell evaluation starts becoming slightly difficult due to increased thickness of the tissue in E) FBS compared with F) rHSA.

Scale bar: 100 µm
Figure 2: Corneal endothelial cells showing significant folds due to the tissue swelling in A) FBS (marked with white dashes) and minimal amount of iatrogenic folds observed in the tissues from B) rHSA (marked with white dashes). At certain points, there were areas with significant amount of damage (marked) observed from the tissues preserved in C) FBS compared with those preserved in D) rHSA. The tissues preserved in E) FBS showed stretched and polymorphic cells (marked) when observed using ZO-1 expression, which is assumed to be at the areas with folds compared with minimal polymorphism observed from the tissues preserved in F) rHSA.
Scale bar: 100 μm