1 **Human corneal endothelial cell assessment from the tissues preserved in**
2 **serum-based and synthetic storage media**
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21 **Running title:**
22 Corneal endothelial cell assessment
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24 **Disclosure:**
25 No conflict of interest exists

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/mm^2 in FBS and 1864 cells/mm^2 in rHSA. Operator 2 counted 1702 cells/mm^2 in FBS and 1858 cells/mm^2 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.

51 **Keywords:**

52 Eye bank; cornea; preservation, organ culture; synthetic medium; recombinant human

53 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.

78 Inter and intra-bank variability has previously been reported[9]. Perfectly aligned cell
79 membrane visualization with alizarin red staining has shown a precision range between
80 +5 and -5%[10]. If the tissues have iatrogenic folds, it increases the difficulty level of cell
81 counts resulting in a significant counting error. The precision, however, is still
82 dependent on the observer. In our earlier report, we showed that tissues preserved in
83 recombinant human serum albumin (rHSA) have better endothelial cell viability and
84 overall tissue quality[11]. This study aims at comparing the endothelial cell visualization
85 of the tissues preserved in the media supplemented with fetal bovine serum (FBS) i.e.
86 Cornea Max® (Eurobio, Les Ulis, France) and rHSA i.e. Cornea Syn® (Eurobio, Les
87 Ulis, France).

88 **Methods:**

89 Ethical statement

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

95

96 Tissue evaluation

97 Morphology (n=48) of the tissues was visualized using Trypan blue stain (0.25%
98 wt/vol) (VisionBlue, D.O.R.C., Zuidland, The Netherlands) to evaluate the percentage
99 of dead/necrotic cells before and after preservation. The endothelium was exposed to a
100 hypotonic sucrose solution, which helps counting the number of endothelial cells and
101 to examine the general morphology (pleomorphism and polymegathism). ECD was
102 counted using a 10X10 reticule (0.1mm^2) attached to the eyepiece of an inverted
103 microscope (Axiovert, Zeiss, Germany) at 100x magnification. ECD was expressed as
104 an average of five different counts, each performed at a different endothelial area.
105 Number of folds were counted manually using the same set of images.

106

107 Alizarin red staining

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

112

113 Immunostaining for tight junctions using zonula occludens-1 (ZO-1)

114 The tissues (n=6, from each condition), after preservation, were washed with PBS and
115 the Descemet membrane-endothelial complex was peeled using a standard stripping
116 technique used for Descemet membrane endothelial keratoplasty (DMEK)[12]. The
117 stripped DMEK tissue was fixed in 4% paraformaldehyde at room temperature (RT)
118 for 20 minutes'. The cells were permeabilized with 0.5% triton x-100 for 30 minutes.
119 After blocking with 5% goat serum for 1 hour at RT, the cells were incubated with
120 primary antibody [anti-ZO-1 (pre-conjugated with FITC), 1:200 (ZO1-1A12, Thermo
121 Fisher Scientific, Rochester, NY, USA)] for 3 hours at RT. After each step, the cells
122 were sequentially washed three times with PBS. The stripped tissues were flat mounted,
123 covered with the mounting medium (Vectorshield, Vector labs, CA, USA) and
124 examined under a Nikon Eclipse Ti-E (Nikon, Burgerweeshuispad, Amsterdam)
125 microscope using the NIS elements software (Nikon).

126

127 Measurements and statistical analysis

128 ImageJ (FIJI) software was used to measure and analyze the data. For ZO-1, the area
129 was selected and a pre-defined command (macros) was used[13,14] that converts the image
130 to overlay masks. Total number of cells were automatically calculated by the software.
131 Whereas, the hexagonal (all 6 borders in each cell) and polymorphic cells were counted
132 manually based on the cell structure in a particular area.

133 Non-parametric Wilcoxon test for paired data and one-way ANOVA test for
134 independent measures were used to check the statistical significance between FBS and
135 rHSA preserved tissues, where $p<0.05$ was deemed statistically significant. A post-hoc
136 correction to the significance was applied using Bonferroni test[13,14].

137 **Results:**

138 General morphology

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

148

149 Alizarin red staining

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

155

156 ZO-1 immunostaining, hexagonality and polymorphism analysis

157 ZO-1 staining showed stretched and polymorphic cells (marked with white dashes) on
158 the tissues preserved in FBS group (Figure 2E). This was not observed from the tissues
159 preserved in rHSA group (Figure 2F). 68.71(±3.82)% hexagonal cells were recorded
160 from the tissues preserved in FBS compared with 70.88(±2.80)% hexagonal cells from
161 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.

<u>Inter and intra-operator variability</u>

ECD counts before preservation from operator 1 between FBS (1885±156 cells/mm^2) and rHSA (1890±159 cells/mm^2) (p=0.9443) and from operator 2 between FBS (1895±167 cells/mm^2) and rHSA (1900±163 cells/mm^2) (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/mm^2) and rHSA (1864±132 cells/mm^2) from operator 1 was found to be significantly different (p=0.0084). ECD values from operator 2 between FBS (1702±101 cells/mm^2) and rHSA (1858±138 cells/mm^2) 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.

186 **Discussion**

187 Data from the European eye banks suggest that approximately 15-20% of the corneas
188 are discarded due to poor endothelial cell counts (usually <2,000 cells/mm^2). Hence,
189 the tissues with borderline cell counts need to be precisely evaluated to primarily
190 categorize it for transplantation or research purpose. This may have a huge impact on
191 increasing or decreasing the number of corneal tissues suitable for transplant. It is
192 important to note that ECD measurement before the tissue is shipped from the eye bank
193 is one of the main parameters of corneal quality control, and therefore needs to be
194 recorded precisely. In a study carried out by French eye banks, more than half of the
195 cell counts showed deviations by more than 10% from their actual counts. The counts
196 were over-estimated by 33% and under-estimated by 26%[14]. This data relates to
197 delivery of poor quality corneas for transplantation purposes in certain centers and an
198 increased discard rate in others[15]. Even computer-aided methods have failed to provide
199 reliable results, mainly due to poor imaging of the cells[15].
200 In this masked, donor-matched study, we observed that visualization of the endothelial
201 cells after preservation of the tissues in rHSA supplemented media was better than those
202 preserved in FBS containing media. This further minimized the endothelial cell
203 counting error. We do not claim that our cell counts were 100% reliable, as this depends
204 mostly on the masked observer and can be subjective. However, as visualization of the
205 endothelial cells from the tissues preserved in rHSA group was better, it reduced the
206 risk of over- or under-estimation of cell counts. It was observed that the cells around
207 the deep folds changed their morphology by stretching, which created difficulties in
208 counting the cells at 100X magnification. An increase in the number of folds and cell
209 stretching escalated the magnitude of cell counting errors, which was not observed in
210 the tissues preserved in rHSA.

211 Cornea Max® is a routinely used OC media that contains FBS, which possibly creates
212 haze due to turbidity, thus affecting the visualization and image quality of the
213 endothelial cells. Cornea Syn® is supplemented with rHSA and could reduce this error
214 significantly, as it allows clear observation and evaluation of endothelial cells.
215 Importantly, better intercellular border visualization helps in reducing the cell counting
216 errors and the overall time required for counting the number of cells, which plays a
217 significant role in the field of eye banking, especially for high volume eye banks. As
218 the cells from FBS could not be evaluated precisely due to the increased number of
219 iatrogenic folds and poor cell visualization, the recorded ECD values were not as high
220 as the tissues from the same donor that was preserved in rHSA post-preservation, thus
221 increasing the chances of over- or under-estimation.
222 A gradual change of ionic content between the cells and the intercellular junctions may
223 increase water egress from the cells, thus promoting dilation of the intercellular spaces
224 in the presence of a hypotonic solution[2]. The endothelial cell visualization after osmotic
225 dilation of the intracellular spaces is usually affected after 2 weeks. However, we
226 observed that the cell visualization remained constant even during the fourth week if
227 the tissues were preserved in rHSA. This visualization improved from day 0 to week 1
228 of the storage and remained relatively constant thereafter up to week 4. However, slight
229 deterioration in the cell visualization was observed with the FBS group during this time
230 because of increasing thickness and generation of folds due to swelling. Endothelial
231 cells from the tissues preserved in FBS were difficult to differentiate, and this may
232 explain a difference in cell counts arising between the two operators. The final quality
233 further improves the precision of cell counts and reduces the overall wastage of tissues
234 deemed for transplantation due to improper endothelial cell counts[6-8,10,16,17].
235 Preserving corneal tissue in Cornea Syn® supplemented with rHSA has shown the

236 following advantages: a) It is fully synthetic and therefore complete deduction of
237 animal or animal derived products is possible; b) It does not require additional batch-
238 to-batch testing for maintaining good standards of practice; c) There is good
239 visualization of the endothelial cells leading to ease of cell counting and reducing the
240 under- or over-estimation, which has a huge impact on the tissue selection procedure;
241 d) It minimizes the extended time period for counting the endothelial cells, and limits
242 the loss of good quality tissues for transplantation; e) It improves the possibility of
243 counting the cells at iatrogenic folds thus reducing the cell count errors due to deep
244 folds; f) It maintains cell viability and; g) as the pre-endothelial cell counts are precise,
245 it allows a more accurate evaluation of post-operative endothelial cell loss.
246 It is known that the preservation media is mostly selected based on economic
247 arguments[2]. As Cornea Syn® contains a recombinant ingredient, the overall cost could
248 be relatively higher than the currently used Cornea Max® media. However, as the
249 European laws favor the 3 'Rs' policy and the advantages of using rHSA are greater,
250 the benefits would compensate the economic cost. We have tested several parameters
251 that are usually required for an eye bank to justify a tissue for transplantation[11], and
252 have found that the rHSA series was effective in statistically compared parameters. The
253 rHSA series therefore may constitute a substantial advancement in the tissue
254 preservation and quality control assessment including ECD measurements[11].

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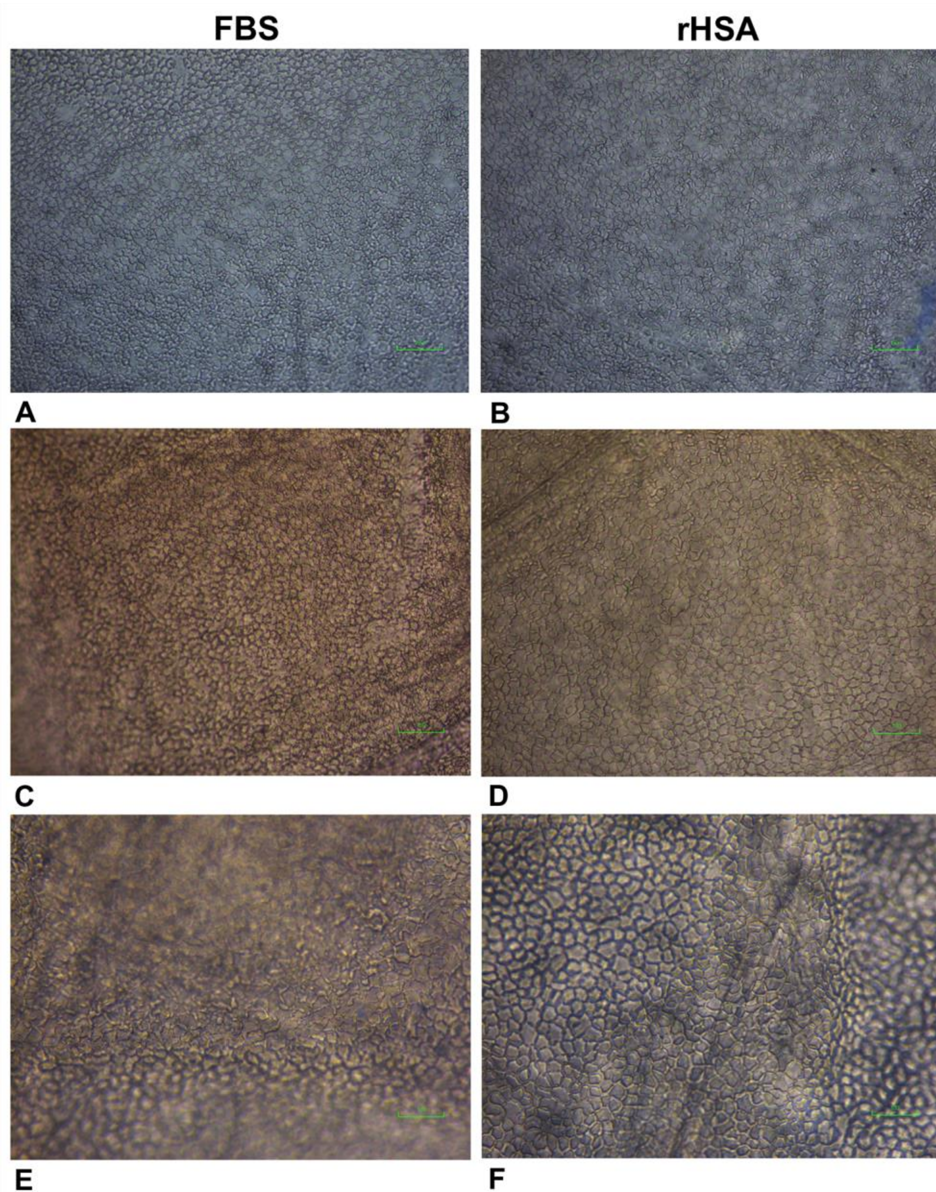
303

304 **Figure legends:**



305

306 **Figure 1:** Corneal endothelial cells as observed before preservation in A) FBS and B)
307 rHSA supplemented media. Endothelial cells from the same tissues observed after
308 preservation at 31°C in C) FBS and D) rHSA. At a higher magnification, the endothelial
309 cell evaluation starts becoming slightly difficult due to increased thickness of the tissue
310 in E) FBS compared with F) rHSA.
311 Scale bar: 100 µm
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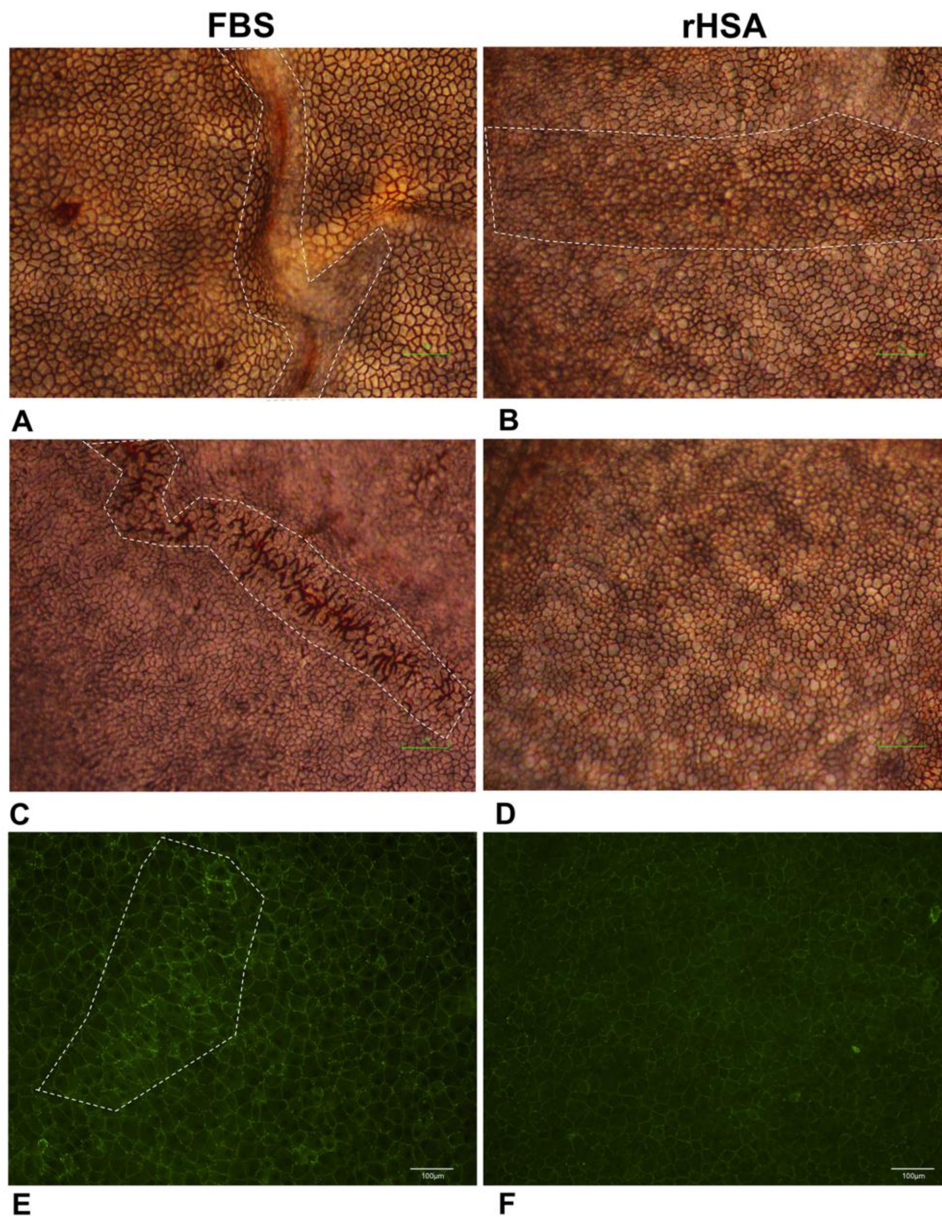

314

315 **Figure 2:** Corneal endothelial cells showing significant folds due to the tissue swelling
316 in A) FBS (marked with white dashes) and minimal amount of iatrogenic folds
317 observed in the tissues from B) rHSA (marked with white dashes). At certain points,
318 there were areas with significant amount of damage (marked) observed from the tissues
319 preserved in C) FBS compared with those preserved in D) rHSA. The tissues preserved
320 in E) FBS showed stretched and polymorphic cells (marked) when observed using ZO-
321 1 expression, which is assumed to be at the areas with folds compared with minimal
322 polymorphism observed from the tissues preserved in F) rHSA.

323 Scale bar: 100 µm