Evaluation of structural changes of the corneal endothelial layer at various lengths of eye bank storage

Ernest D. Kovarik
University of Nebraska Medical Center

Follow this and additional works at: https://digitalcommons.unmc.edu/mdtheses

Part of the Medical Education Commons

Recommended Citation
Kovarik, Ernest D., "Evaluation of structural changes of the corneal endothelial layer at various lengths of eye bank storage" (1964). MD Theses. 23.
https://digitalcommons.unmc.edu/mdtheses/23

This Thesis is brought to you for free and open access by the Special Collections at DigitalCommons@UNMC. It has been accepted for inclusion in MD Theses by an authorized administrator of DigitalCommons@UNMC. For more information, please contact digitalcommons@unmc.edu.
THE EVALUATION OF STRUCTURAL CHANGES OF THE CORNEAL ENDOTHELIAL LAYER AT VARIOUS LENGTHS OF EYE BANK STORAGE

Ernest Delano Kovarik

Submitted in Partial Fulfillment for the Degree of Doctor of Medicine
College of Medicine, University of Nebraska
February 4, 1964
Omaha, Nebraska
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Endothelium, a Metabolic Pump</td>
<td>1</td>
</tr>
<tr>
<td>The Necessity of a Viable Endothelium in</td>
<td>2</td>
</tr>
<tr>
<td>Corneal Grafts</td>
<td></td>
</tr>
<tr>
<td>Earlier Work Evaluating Effects of Cold</td>
<td>4</td>
</tr>
<tr>
<td>Storage on the Endothelium</td>
<td></td>
</tr>
<tr>
<td>Hypothesis</td>
<td>5</td>
</tr>
<tr>
<td>II. REPORT OF STUDY</td>
<td>6</td>
</tr>
<tr>
<td>Material and Methods Used</td>
<td>6</td>
</tr>
<tr>
<td>III. RESULTS</td>
<td>12</td>
</tr>
<tr>
<td>Controls</td>
<td>12</td>
</tr>
<tr>
<td>Six Hour Storage</td>
<td>15</td>
</tr>
<tr>
<td>Twelve Hour Storage</td>
<td>16</td>
</tr>
<tr>
<td>Twenty-four Hour Storage</td>
<td>16</td>
</tr>
<tr>
<td>Forty-eight Hour Storage</td>
<td>17</td>
</tr>
<tr>
<td>Seventy-two Hour Storage</td>
<td>18</td>
</tr>
<tr>
<td>Ninety-six Hour Storage</td>
<td>18</td>
</tr>
<tr>
<td>One-Hundred-Twenty Hour Storage</td>
<td>19</td>
</tr>
<tr>
<td>IV. DISCUSSION AND CONCLUSIONS</td>
<td>20</td>
</tr>
<tr>
<td>Vacuolation</td>
<td>20</td>
</tr>
<tr>
<td>Cellular Staining</td>
<td>22</td>
</tr>
<tr>
<td>Cellular Death</td>
<td>23</td>
</tr>
<tr>
<td>Imbibition</td>
<td>23</td>
</tr>
<tr>
<td>Suggestions for Improvement</td>
<td>24</td>
</tr>
<tr>
<td>Conclusions</td>
<td>25</td>
</tr>
<tr>
<td>V. SUMMARY</td>
<td>27</td>
</tr>
<tr>
<td>VI. ACKNOWLEDGEMENTS</td>
<td>29</td>
</tr>
<tr>
<td>VII. BIBLIOGRAPHY</td>
<td></td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Sequence of Eye Storage Time from 6 to 120 Hours Summarizing the Corneal Appearance, Corneal Thickness, Endothelial Intactness, and Macroscopic and Microscopic Staining Appearance in Comparison with Their Controls</td>
<td>13</td>
</tr>
<tr>
<td>II. Comparison of Cytoplasmic Vacuolation of the Experimental Stored Eye with its Control</td>
<td>14</td>
</tr>
</tbody>
</table>
THE EVALUATION OF STRUCTURAL CHANGES
OF THE CORNEAL ENDOTHELIAL LAYER AT VARIOUS LENGTHS
OF EYE BANK STORAGE

I. INTRODUCTION

With the onset of eye bank procedures, the need for preservation of corneal tissue for more than three days has been almost eliminated. At the present time, most eye bank storage procedures consist of keeping the eye at four degrees centigrade in saline vapor. It is now generally accepted that penetrating grafts should be used within twenty-four hours (32). If the corneas are held for a longer period under the saline vapor technique, a penetrating transplant should be done with reluctance (30).

It is the purpose of this study to substantiate the early use of donor material in cases of perforating grafts.

Endothelium, a Metabolic Pump

Living corneas must be maintained in the deturgesced state in order to maintain normal corneal transparency. Any change or alteration in the mechanism that maintains or controls excessive corneal stromal imbibition will lead to serious opacification of the cornea and, in turn, will lead to interference of visual clarity.
Much work has been done to explain the exact mechanism governing corneal permeability and prevention of corneal edema. As far back as 1873, Leber (14) demonstrated that an intact endothelium was necessary to prevent corneal aqueous infiltration under pressure.

The well reputed osmotic theory of Cogan and Kinsey (4), necessitating an intact set of semi-permeable endothelial and epithelial membranes for maintenance of corneal transparency, has now been replaced by Harris' (10) (11) theory of a metabolic pump activated by epithelial and endothelial cells. It has been demonstrated more recently that only the endothelium is capable of pumping out fluid by active metabolism, thereby maintaining deturgescence (10) (17).

The Necessity of a Viable Endothelium in Corneal Grafts

The loss of viability of the endothelium plays an important role in giving good results of full thickness corneal grafts (15). Stocker (28), in order to establish immediate influence of a graft with a partial deficiency of the endothelium, grafted a cornea into a rabbit that had one-half of the endothelium scraped off. Four days following the operation, microscopic examination revealed much swelling of the denuded half of the graft as compared with the intact
half. He also presented a case of endothelial detachment from the grafted tissue which developed into an edematous and thickened graft. These observations indicate that an intact and viable endothelium appears to be of paramount importance and is necessary to prevent post-operative transplant alterations.

Review of the fate of keratoplasty reveals that much work has been done in proving whether the original endothelium of the graft survives without replacement by the host following transplant, or whether delayed replacement occurs.

Espiritu and co-workers (7), using sex chromatin studies, showed that rabbit endothelial cells persist for a period of four months followed by a gradual replacement. After a period of seven months the host replaced almost all the graft endothelial cells. P. K. Basu (2), also using sex chromatin as a biologic cell marker, studied cat corneal transplants. Findings revealed that for a period of at least three months, the sex characteristics of a graft remained unaltered. More recent work in which rabbit donor material was labeled with tritiated thymidine reveals that cells of successful corneal homografts may persist indefinitely (9) (20) (22).

Although the fate of these studies cannot be directly related to human corneal endothelial grafts, it does indicate that the cells'
intactness and viability are prime necessities since they must survive and function for at least several weeks post transplant. Without a functional and viable endothelium, keratoplastic complications are evident (24).

To reiterate, the purpose of this study was to substantiate the early use of donor material for perforating grafts and to correlate corneal endothelial cell changes with various lengths of eye storage under eye bank conditions.

Earlier Work Evaluating Effects of Cold Storage on the Endothelium

Stocker (28) proceeded to evaluate the effects of various lengths of cold storage upon the endothelium. He demonstrated that human corneas, after twenty-four hours storage, had an identifiable endothelium but with indistinct cells of a shriveled appearance. At forty-eight hours, it was found that the endothelial layer was no longer continuous and showed extensive vacuolation of the cytoplasm and nuclei. At ninety-six hours, the endothelial layer was found to be absent over large areas.

Similar work with human corneas reveals endothelial cell changes consisting of progressive shrinkage and vacuole formation related to time in storage (23).
Hypothesis

Even though the epithelium and endothelium have a protective function, a slow seepage of fluid into the stroma continues. As stated earlier, it is the endothelium which actively maintains a clear cornea. When the ability to remove fluid is abolished, access imbibition will develop, thus causing a thickened and cloudy cornea (17).

It was therefore hypothesized that microscopic endothelial changes would correlate with opacification and corneal swelling. In other words, the earliest change noted microscopically should correspond with onset of opacity and swelling. As storage time progresses, greater disruption of endothelium should occur due to degenerative processes, and should correspond with increased cloudiness and swelling of the cornea due to increased imbibition.
II. REPORT OF STUDY

In order to detect the earliest changes possible, each experimental eye was morphologically compared to its pair, the control. Three criteria were established:

1. Each endothelial layer was compared to its control with regard to cellular and cytoplasmic morphology and intactness.

2. The earliest gross evidence of corneal opacity was recorded.

3. During preparation for mounting, a comparison of corneal thickness was made to its control.

Materials and Methods Used

Rabbit corneas of a mixed strain were used. Rabbits were those used for the Freidman pregnancy test. Each was approximately 8-10 weeks old. All external conditions such as housing, diet and room temperature were kept as constant as possible. The eyes were enucleated at time of sacrifice for obtaining the ovaries and tubes for the pregnancy test. Therefore, each rabbit had been injected approximately forty-eight hours prior to killing with either urine or serum. These animals were all sacrificed in the afternoon by ear marginal vein injection of air.

Enucleation was done one to three minutes following death with as much care as possible to prevent any accidental trauma to
either the epithelium or endothelium, distortion of or increased pressure to the globe which might interfere with the normalcy of the endothelial layer. Each eye was examined at this time for gross defects and transparency of the cornea. Fourteen pairs of eyes were examined in the present study.

Upon removing the eyes, one of the pair was always used as a control and was placed immediately into fixative, while the other eye was placed whole into 2 cc. of sterile normal saline in a 50 cc. container. Two drops of neosporin ointment were placed on the cornea prior to storage in 2 cc. saline. The fixative used was fresh 10% formalin buffered with sodium bisulfate. All corneas, when placed into fixative, were isolated by transecting the bulb 4-5 mm behind the limbus, removing the lens, vitreous, ciliary body and iris and then immersed with their circular scleral tags into 50 cc. of formalin. This enables rapid contact of fixative with the endothelial layer. Again, caution was taken to prevent trauma to the endothelial membrane. The isolated corneas with scleral rim were kept in fixative at least 12 hours before staining. Both control and experimental paired eyes were placed in a refrigerator at approximately four degrees centigrade within 15-45 minutes following enucleation.

With this procedure a control corneal endothelial layer is ob-
tained which is immediately fixed. This should represent morphologically a normal appearing and intact endothelium to be correlated and compared with its experimental pair. Gross comparisons as to edema and opacification can be made in this same manner.

Eyes were stored under eye bank conditions for 6, 12, 24, 48, 72, 96 and 120 hours. Following storage, each cornea was removed and placed in formalin as described above.

Following fixation of at least twelve hours, the cornea with its scleral ring was washed in distilled water for 3-5 minutes and then stained by a modified Hematoxylin-Eosin technique as follows:

1. Hematoxylin 8-10 minutes followed by a rinse in distilled water.
2. Dip two times in acid-alcohol.
3. Dip twice in two changes of 95% alcohol.
4. Place in 1% Lithium Carbonate until the cornea turns deep blue.
5. Rinse again in distilled water.
6. Place in Eosin solution for 1-2 minutes.
7. Wash in two changes of 95% alcohol.
8. Wash in absolute alcohol and, finally,
9. Two changes of xylol.
The cornea was not kept in xylol over three minutes before mounting. The stained cornea, with xylol in the concavity, was placed with the epithelial surface on the slide and observed under the microscope. In this manner it is possible to scan and observe the entire endothelial surface and thereby evaluate its intactness, morphological cellular integrity and any disruption that may have occurred during preparation. A microscope with high light intensity is necessary in order to observe the endothelial structure through the entire corneal thickness.

Since the endothelium is a single flat layer at the posterior surface of the cornea, morphologic studies by routine histologic procedures are inadequate. Flat preparations, secured in earlier work either by a modified method used by Stocker (28) and von Sallmann (31) or by serial sectioning, were found to be inadequate. Stripping of the endothelial layer introduces artificial disruptions and possible changes, while serial sectioning does not allow for large enough sections or equally representative areas of the cornea to be adequately studied. Therefore, the following technique was used in order to observe the intact cornea in its entirety and to insure a uniform representation of the cornea at mounting.

Strips of cornea and scleral tags measuring 2-4 mm were cut out across the entire cornea diameter with a razor blade. This
now produced a strip consisting of a well represented piece of cornea from which the central as well as peripheral endothelium could be evaluated. With the epithelial surface on a dissecting board, the scleral tag was held firmly with small, fine-toothed forceps and with a new razor blade, the cornea was bisected a few mm away from the corneal scleral junction. This bisection was accomplished by cutting at an angle toward the sclera, thus undercutting at an angle through the stroma. Then, by continuing to hold the tag with the forceps, another bisection was made, this time beginning at the corneal scleral junction cutting an angle away from the sclera through the corneal scleral meshwork. This produced a piece of cornea with tapered ends of only endothelium, Descement's membrane and various thicknesses of stroma. This procedure was repeated on the central section, thus giving good representation. Many pieces from the remainder of the cornea can be prepared in the same way. After each piece was obtained, it was necessary to immediately place it on a slide with another slide covering it in order to facilitate flattening. It was found to be beneficial to place a light weight on the slide.

This procedure eliminates many of the folds and wrinkles obtained in flat stripping preparations and from too much drying which
has proven to cause considerable shrinkage. In the procedure described, one gets areas thin enough for high power magnification observation, especially at the undercut area without introducing error.

The isolated pieces, after being flattened, were mounted with endothelium up, epithelium and stroma down on the slide. Preparations were mounted with Permount. The microscopic examination was carried out shortly following mounting in order to prevent the influence of fading or bleaching on the final observation.
III. RESULTS

Relatively good results were obtained in this series of eye storage experiments. Macroscopically all corneas were clear at time of enucleation. Trauma to the endothelium occurred only once during the preparation of the controls by the accidental scratching of the endothelial surface with scissors. Enough of the cornea was undamaged to give good reliable results. (The results of this experiment are summarized in Tables I and II herein).

Controls

Fourteen pairs of eyes were examined. One cornea of each pair was used as a control and the other was examined at various lengths of cold storage. All the control corneas stained uniformly dark purple macroscopically, and only two of the controls resulted in somewhat poorer microscopic cellular clarity.

In only four controls was a "normal" endothelial mosaic structure found, that is, a granular protoplasm with a centrally located, kidney bean-shaped nuclei without cytoplasmic vacuoles (28).

The remaining controls revealed various gradations of cytoplasmic and occasional nuclear vacuolations. The cells were pyknotic in appearance with strands of cytoplasmic material streaming from
TABLE I

SEQUENCE OF EYE STORAGE TIME FROM 6 TO 120 HOURS
SUMMARIZING THE CORNEAL APPEARANCE, CORNEAL THICKNESS,
ENDOTHELIAL INTACTNESS, AND MACROSCOPIC AND MICROSCOPIC STAINING APPEARANCE IN COMPARISON WITH THEIR RESPECTIVE CONTROLS

<table>
<thead>
<tr>
<th>Hrs. in Storage</th>
<th>Gross Corneal Appearance</th>
<th>Increased Corneal Thickness</th>
<th>Intact Endothelium</th>
<th>Macroscopic Staining Appearance</th>
<th>Microscopic Staining Appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 Hrs.</td>
<td>Clear</td>
<td>None</td>
<td>Yes</td>
<td>Good</td>
<td>Good **</td>
</tr>
<tr>
<td>12 Hrs.</td>
<td>Clear</td>
<td>None</td>
<td>Yes</td>
<td>Good</td>
<td>Good **</td>
</tr>
<tr>
<td>24 Hrs.</td>
<td>Clear</td>
<td>None</td>
<td>Yes</td>
<td>Good</td>
<td>Good</td>
</tr>
<tr>
<td>24 Hrs.</td>
<td>Clear</td>
<td>None</td>
<td>Yes</td>
<td>Fair</td>
<td>Fair</td>
</tr>
<tr>
<td>24 Hrs.</td>
<td>Clear</td>
<td>None</td>
<td>Yes</td>
<td>Good</td>
<td>Good **</td>
</tr>
<tr>
<td>48 Hrs.</td>
<td>Clear</td>
<td>None</td>
<td>Yes</td>
<td>Good</td>
<td>Poor</td>
</tr>
<tr>
<td>48 Hrs.</td>
<td>Clear</td>
<td>None</td>
<td>Yes</td>
<td>Good</td>
<td>Good **</td>
</tr>
<tr>
<td>48 Hrs.</td>
<td>Clear</td>
<td>None</td>
<td>Yes</td>
<td>Good</td>
<td>Good **</td>
</tr>
<tr>
<td>72 Hrs.</td>
<td>Opacity</td>
<td>None</td>
<td>No *</td>
<td>Fair</td>
<td>Poor</td>
</tr>
<tr>
<td>72 Hrs.</td>
<td>Opacity</td>
<td>Thickening</td>
<td>No *</td>
<td>Poor</td>
<td>Poor</td>
</tr>
<tr>
<td>96 Hrs.</td>
<td>Opacity</td>
<td>Thickening</td>
<td>No *</td>
<td>Poor</td>
<td>Poor</td>
</tr>
<tr>
<td>120 Hrs.</td>
<td>Opacity</td>
<td>Thickening</td>
<td>No *</td>
<td>Poor</td>
<td>Poor</td>
</tr>
</tbody>
</table>

* Non-viable cells lacking intercellular cohesion
** Stained slightly less distinctly than respective controls
TABLE II.
COMPARISON OF CYTOPLASMIC VACUOLATION OF THE EXPERIMENTAL STORED EYE WITH ITS CONTROL

<table>
<thead>
<tr>
<th>Hrs. in Storage vs. Paired Control</th>
<th>Cytoplasmic Vacuoles</th>
<th>Hrs. in Storage vs. Paired Control</th>
<th>Cytoplasmic Vacuoles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 - F *</td>
<td>++</td>
<td>8 - F</td>
<td>None</td>
</tr>
<tr>
<td>1 - 6</td>
<td>Rare</td>
<td>8 - 48</td>
<td>++++</td>
</tr>
<tr>
<td>2 - F</td>
<td>+++</td>
<td>9 - F</td>
<td>+</td>
</tr>
<tr>
<td>2 - 12</td>
<td>Rare</td>
<td>9 - 48</td>
<td>Not Visualized **</td>
</tr>
<tr>
<td>3 - F</td>
<td>+</td>
<td>10 - F</td>
<td>++</td>
</tr>
<tr>
<td>3 - 12</td>
<td>+</td>
<td>10 - 72</td>
<td>Multiple Minute Vacuoles ***</td>
</tr>
<tr>
<td>4 - F</td>
<td>++</td>
<td>11 - F</td>
<td>+</td>
</tr>
<tr>
<td>4 - 24</td>
<td>++++</td>
<td>11 - 72</td>
<td>Multiple Minute Vacuoles ***</td>
</tr>
<tr>
<td>5 - F</td>
<td>+</td>
<td>12 - F</td>
<td>++</td>
</tr>
<tr>
<td>5 - 24</td>
<td>++</td>
<td>12 - 72</td>
<td>Multiple Minute Vacuoles ***</td>
</tr>
<tr>
<td>6 - F</td>
<td>None</td>
<td>13 - F</td>
<td>None</td>
</tr>
<tr>
<td>6 - 24</td>
<td>++</td>
<td>13 - 96</td>
<td>Multiple Minute Vacuoles ***</td>
</tr>
<tr>
<td>7 - F</td>
<td>++</td>
<td>14 - F</td>
<td>None</td>
</tr>
<tr>
<td>7 - 48</td>
<td>++++</td>
<td>14 - 120</td>
<td>Multiple Minute Vacuoles ***</td>
</tr>
</tbody>
</table>

* F = Immediately fixed control
** Distinct intercellular staining without cytoplasmic and nuclear visualization
*** Non-viable ghost-like cells surrounded by multiple minute granular appearing vacuoles causing disruption of intercellular cohesion

- 14 -
the nucleus, which seemed to be due to vacuole displacement. Although the nuclei were centrally located in the cells, the uniform kidney bean-shaped nucleus was not found, but instead appeared round to elongated with various bizarre shapes together with an irregular perinuclear margin. A few nuclei gave the appearance of "pre eruption". Due to the marked vacuolation, protoplasmic granularity was not observable as seen in the "normal" endothelial picture.

All the control corneas revealed an intact, single-celled endothelial layer upon Descement's membrane. There seemed to be no great variation or transition between central and peripheral endothelial cells except that at the periphery, the nuclei appeared somewhat more elongated with perhaps slightly less cytoplasmic vacuolation.

Six Hour Storage

One eye was placed under eye bank storage procedures for six hours. At the end of six hours the cornea was grossly clear. There was no apparent increase in corneal thickness, and staining following fixation was macroscopically uniform and comparable with its control. Microscopic examination disclosed slightly fainter cellular staining but an intact endothelium. The nuclear configuration was
that of a normal kidney bean-shape with possibly an increase in size. However, an increase in size was quite difficult to compare since the paired control was one of pyknosis and vacuolation. The six-hour eye revealed no nuclear and only rare cytoplasmic vacuolation. There were a few darkly stained and shrunken nuclei found throughout, with a large halo-like vacuole circumscribing the nucleus. Large aggregates of these types of cells were not found.

**Twelve Hour Storage**

Two eyes were stored for twelve hours in saline. Cloudiness of the cornea was not apparent grossly at twelve hours and no corneal thickening was evident. The tissue stained well macroscopically. Microscopic examination revealed fainter staining of cellular morphology compared to the controls but an intact endothelium was found in both. There was a tendency for the nuclei to be more swollen and bean-shaped compared to the pyknotic and vacuolated controls. Fewer vacuoles were evident; in fact, one of the endothelial layers had almost no vacuolation. In general, a more compact cellular appearance was present with an occasional grouping of darkly stained and shrunken nuclei.

**Twenty-Four Hour Storage**

Three eyes were stored at twenty-four hours saline storage. At twenty-four hours, corneal cloudiness was not noted grossly and,
again, no corneal increase in thickness was apparent. One of the
three endothelial layers stained well both macroscopically and micro-
scopically. However, the cellular morphology of the other two
stained less distinctly than their controls with one even staining
lightly macroscopically. All three corneas revealed an intact
endothelium. Increased vacuolation was noted compared to their
respective paired controls. Vacuolation was so great in one experi-
mental cornea that strands seen in its control were not observable
due to the large vacuoles formed. In two corneas many more hea-
vily stained, shrunken nuclei with halo-like vacuoles were present
than at six and twelve hour corneal storage.

Forty-Eight Hour Storage

At forty-eight hours three corneas revealed no cloudiness or
apparent increased corneal thickness. Macroscopically all three
appeared to be stained well and uniformly, however, microscopic
staining was poor to good. The endotheliums of all three corneas
were intact. The intercellular material of one layer stained well out-
lining a normal, intact, hexagonal configuration without cytoplasmic
and nuclear staining. An increase in vacuole size and number was
noted giving rise to less stranding. Increased swelling of nuclei
in comparison to their controls was apparent. Shrunken and darkly
stained nuclei were scattered in strips and various aggregates throughout the endothelium, but an actual increase in quantity compared to those found at twelve and twenty-four hours was difficult to evaluate subjectively. Vacuoles surrounding some of the shrunken nuclei were present.

**Seventy-Two Hour Storage**

Three eyes stored at seventy-two hours grossly revealed the onset of corneal cloudiness. Two corneas, on sectioning, were noted to be thickened and lightly stained macroscopically. A third cornea demonstrated no increase in thickness and stained grossly comparable to its control. Microscopic cellular staining of all three was markedly poor. Cells appeared as "ghost cells" and actual morphologic examination was not possible although extremely swollen and pale nuclei were apparent. Actual endothelial tears or areas of disruption were not evident but "cracks", multitudes of tiny vacuoles and granular-like material outlined each cell displaying loss of intercellular cohesion.

**Ninety-Six Hour Storage**

At ninety-six hours corneal opacity and corneal thickening of a grade more than at 72 hours was visible. The cornea was boggy in nature and stained lightly. Microscopically actual endothelial dis-
ruptions were not visualized. Very poor cellular staining was evident. Nuclei were markedly swollen with multitudes of tiny vacuoles and "cracks" outlining the degenerated cells.

One-Hundred-Twenty Hour Storage

At one-hundred-twenty hours corneal opacity and thickening, a grade more than at ninety-six hours, were visible. The cornea was very boggy and macerated. Staining was very poor. Actual endothelial tears or disruptions were not found but "ghosts" were again noted depicting the degenerated cells.
IV. DISCUSSION AND CONCLUSIONS

Vacuolation

In the usual process of histo fixation, extremely rapid post mortem vacuolation occurs which quickly produces distortion of the endothelial cells (8) (25). In this experiment more than half of the control eyes were found with protoplasmic and nuclear vacuolation revealing a pyknotic, strand-like appearance.

Speakman (25), using a more rapid fixing technique on cat and rabbit eyes, has demonstrated preservation of an intact, hexagonal cell arrangement without vacuolation. Ludwig von Sallman and collaborators (31) fixed the endothelium while the animals were alive. Degenerative changes such as vacuolation or pyknosis were not observed by this technique in the endothelium of the 2–3 year old rabbits. The prevention of rapid degeneration was thus demonstrated.

It appears that anoxia and other causes, due to cessation of circulation, are responsible for this rapid degeneration and changes in the structure of the cytoplasm. Since vacuolation is considered a degenerative process (degenerative meaning a change from the normal in-vivo state rather than an artifact (8)), fixation prior to animal sacrifice, similar to von Sallmann's work, is indicated to prevent vacuole formation. Vacuole formation does not indicate the cells are no longer
viable, and such a condition does not necessarily detract from the cornea's usefulness.

Stocker (28) and Schaeffer (23) were able to demonstrate progressive increase in vacuolation of human endothelial cells related in time to storage. My results suggest, but do not definitely demonstrate, an absolute progressive increase in vacuolation. The series should be enlarged to ascertain a more definite storage time comparison.

Except for the six- and twelve-hour stored eyes, a progressive increase in vacuolation was observed between each experimental eye and its control. In the case of the six- and twelve-hour eyes, possibly there was less atmospheric exposure to the endothelial layer of the experimental eye in transfer of the cornea to formalin.

There is evidence that much individual variation exists among eyes in the ability of the endothelial layer to withstand the autolytic processes that occur after each eye has been removed and stored. The physical condition of the endothelial cells at time of death may play an important role.

In my work, an increase in vacuole size, in addition to an increase in number, was evident at 48 hours. A cessation of progressive vacuole formations was observable at 72 hours. At this point, the endothelial cells were non-viable and completely deteriorated.
Cellular Staining

Another form of degenerative change was the darkly stained and shrunken nucleus surrounded by a continuous halo-like vacuole. These degenerative cells were not found in any of the controls and only a subjective evaluation indicates a progressive increase in these cells with storage. Actual counts were not made. These nuclei are most likely a condensation of chromatin due to shrinkage and are not an absolute increase in chromatin (DNA).

At 72 hours and beyond, indistinct cellular morphology was observable without the presence of darkly stained and shrunken nuclei. Markedly swollen and refractile nuclei were present without endothelial tears or disruptions. Surrounding the cells were many tiny vacuoles appearing granular in nature with "cracks" at the circumference forming loss of intercellular cohesion. The entire endothelial pattern appeared intact with a cellular make-up best described as "ghost-like".

The disruption and loss of endothelial cells at 96 hours, as described by Stocker (28), and the complete absence at 120 hours, may be due to the technique he used (stripping the layer from the stroma support). Disruption and discontinuity of the endothelium were not demonstrated in my experiment due to the use of the whole corneal technique.
Cellular Death

All changes in cytoplasmic and nuclear structures which suffer from post mortem autolysis seem to cease someplace between 48 and 72 hours. This is indicative of cellular death since prior to 72 hours progression of cellular activity or changes were observable with cessation apparent at and beyond 72 hours.

Imbibition

Beginning at six hours, there was microscopic evidence of corneal imbibition of water demonstrated by lighter cellular staining. This indicated cellular and connective-tissue hydration. Consistent with nuclear fluid uptake, another criteria noted at this time was the slightly larger appearance of the nuclei. Imbibition was not evident macroscopically until 72 hours; at that time early haziness or opacification of the cornea had its onset, together with evidence of increased corneal thickness. Macroscopic staining, in general, was noted to be markedly pale.

Following 72 hours, progressive corneal thickness and cloudiness were observed, indicating rapid corneal hydration. This marked corneal hydration is due to a damaged or non-viable endothelium (18). Imbibition takes place not only via the anterior chamber but also through the epithelium, and at the periphery of the cornea from scleral hydration (5) (6).
The fact remains that the onset and marked progression of opacification and edema of the cornea beyond 72 hours exists and correlates with the microscopic cessation of cellular viability. Thus it is apparent that the endothelium plays an important part in maintaining dehydration since, up to some point between 48 and 72 hours, a degenerating but "viable" endothelium maintained relatively good dehydration. The loss of cellular viability is comparable to an absent endothelium (28) (24).

Suggestions for Improvement

It comes to mind that the distortion and swelling effect of hydration may, in itself, enhance the endothelial membrane's death. The prevention of tumefaction would more than likely prolong the endothelial integrity and viability. It would be interesting to place these eyes into solutions that were more isotonic, thereby preventing corneal or eye tissue hydration and swelling. Since optimum conditions for preserving the viability of different types of cells or tissues are not identical, consideration should be given to establishing an optimum environment to give greatest viability for the cornea as a whole, and the endothelium in particular.

It would also be interesting to have a more accurate indication as to endothelial cellular viability. Perhaps tissue cultures at the
various lengths of eye bank storage or the cessation of mitotic cell division could be introduced as an index. Similar work has been done by Stocker (27) (29) revealing good correlation of grafts with tissue culture results and storage.

Conclusions

In conclusion, this experiment has shown microscopically, progressive endothelial degeneration between each experimental eye and its control. The presence of vacuoles found in the controls demonstrates the rapidity of endothelial alterations and the need for pre-death fixation.

At 72 hours an abrupt morphologic cessation of viability was demonstrated which correlated well with onset of grossly visible corneal opacification and corneal thickening due to marked increase of corneal edema. At 96 and 120 hours progressive corneal cloudiness and swelling were shown. The transition of viable to a non-functional endothelium takes place sometime between 48 and 72 hours. Whether the death of the endothelium is the sole factor for the marked increase in corneal imbibition has not been determined, but correlates well with the denatured appearance of the endothelium, which undoubtedly is the major controlling factor. A damaged or "dead" endothelial layer is highly permeable, therefore leading to a marked edematous cornea.
If homografts of corneal tissue are to become and remain clear, they must be equipped with an inner covering of living endothelial cells (15) (24). Therefore, in view of the results of this paper, one could probably transplant with success up to 48 hours. It is questionable that keratoplasty be attempted beyond this since at some period between 48 and 72 hours, viability is no longer present.

It is rational that the "healthier" and "livelier" the endothelium, the more successful the graft. My conclusion is that donor tissue should be used prior to 24 hours storage and, for best endothelial viability, within 6 to 12 hours.

It cannot be implied that rabbit eye results should be expected of human eyes nor should there be an interchange of conclusions with human corneas. However, basically, it has been shown that the same processes do occur (23) (28) with the same importance in demanding a viable, functioning endothelium, only obtainable early under present saline vapor eye bank storage.
V. SUMMARY

The corneal endothelial layer plays a key role in maintaining corneal deturgescence and dictating the fate of full-thickness keratoplasty. The viability and condition of the endothelium must be such at time of transplant that irreversible deterioration has not yet been reached. It was therefore the purpose of this paper to substantiate the early use of donor corneas by correlating endothelial structural changes with time, at various lengths of storage under eye bank conditions. A correlation was made between morphologic endothelial alterations, onset of gross corneal opacity and corneal thickness.

Fourteen pair of rabbit corneas were used in this experiment. Each pair of eyes was used as a set with one eye stored under eye bank conditions and the other fixed as a control immediately following enucleation. Storage times were 6, 12, 48, 72, 96, and 120 hours. Flat endothelial preparations were made and stained with Hematoxylin and Eosin.

Vacuole formation was demonstrated in control corneas indicating rapid endothelial changes following death. Progressive vacuolation was evident between control and its respective stored eye, revealing progressive degeneration within pairs.
Cessation of viable endothelium was apparent between 48 and 72 hours. A non-viable endothelium was represented by markedly swollen, "ghost-like" cells with lack of intercellular cohesion. Excessive imbibition of corneal tissue correlated well with and beyond the "death" of the endothelium by progressive increase in corneal opacity, thickness and poor staining.
VI. ACKNOWLEDGEMENTS

I am greatly indebted to my advisor, Dr. John C. Filkins, for many helpful suggestions and assistance given to me during the preparation of this paper.

I am also grateful to the staff and employees of the Clarkson Hospital Laboratories for their supply of experimental eyes and technical assistance; to the Department of Ophthalmology, University of Nebraska College of Medicine, for providing equipment and space for carrying out this experiment; and to Lucy Lipp of the College of Medicine's Ophthalmology Pathology Department, for her technical assistance.
VII. BIBLIOGRAPHY


