Effect of the ophthalmic preservative thimerosal on rabbit and human corneal endothelium

Diane L. Van Horn, Henry F. Edelhauser, Gary Prodanovich, Richard Eiferman, and Harlan J. Pederson

Widespread use of the mercurial-containing preservative thimerosal as an antibacterial agent in ophthalmic drugs and solutions warranted an investigation into its possible cytotoxic effects on the functional and ultrastructural integrity of the corneal endothelium. No changes in corneal thickness were observed during 5 hours’ perfusion of the endothelium of rabbit and human corneas with 0.0001 and 0.0005 percent thimerosal in glutathione bicarbonate Ringer’s solution (GBR). Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) of the endothelium of the 0.0001 percent group revealed normal ultrastructure. SEM and TEM of the endothelium of corneas perfused with 0.0005 percent thimerosal for 5 hours revealed condensed mitochondria, cytoplasmic vacuoles, and cytoplasmic flaps at the apical end of the cellular junctions. Perfusion of higher concentrations (0.001 and 0.005 percent) of thimerosal in GBR resulted in increases in corneal thickness after 2 hours and irreversible ultrastructural changes.
damage to the endothelial cells by 5 hours. Corneas perfused with 0.01 and 0.1 percent
thimerosal in GBR showed a rapid and immediate increase in corneal thickness and endo-
thelial cell death and necrosis within 1 hour. It is postulated that the mercury in thimerosal
becomes bound to the cell membrane protein sulfhydryl groups, causing an increase in cellular
permeability. These results suggest that the prolonged exposure of the corneal endothelium
to thimerosal in the accepted antimicrobial dosage of 0.005 to 0.001 percent may result in
functional and structural damage to the endothelium.

Key words: cornea, corneal endothelium, preservatives, scanning and transmission
electron microscopy, thimerosal, toxicity.

Table I. Concentrations of thimerosal
perfused to rabbit corneal endothelium

<table>
<thead>
<tr>
<th>gm./100 ml.</th>
<th>Molar</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0001</td>
<td>2.47 x 10^-5</td>
</tr>
<tr>
<td>0.0005</td>
<td>1.34 x 10^-5</td>
</tr>
<tr>
<td>0.001</td>
<td>2.47 x 10^-5</td>
</tr>
<tr>
<td>0.005</td>
<td>1.34 x 10^-4</td>
</tr>
<tr>
<td>0.01</td>
<td>2.47 x 10^-4</td>
</tr>
<tr>
<td>0.05</td>
<td>1.34 x 10^-4</td>
</tr>
<tr>
<td>0.1</td>
<td>2.47 x 10^-4</td>
</tr>
</tbody>
</table>

The purpose of this investigation was to
close the toxic effects of various concen-
trations of thimerosal on the corneal
endothelium of rabbits and human beings
during in vitro perfusion.

Materials and methods

Paired eyes from albino rabbits (2 to 3 kg.) and
human eyes obtained from the Wisconsin Lions
Eye Bank were used in this study. The corneas

were mounted in a dual-chambered specular micro-
scope which permits constant perfusion of the
endothelium, sequential measurements of corneal
thickness, and continuous observation of the
mosaic-like pattern of the endothelial monolayer.8
The corneas were perfused at 37° C. and 15 mm.
Hg pressure with glutathione bicarbonate Binger’s
(GBR) containing various concentrations of
thimerosal (Table I). The paired control cornea
was simultaneously perfused with GBR alone.

At the end of each perfusion, the corneas were
fixed for transmission electron microscopy (TEM)
and scanning electron microscopy (SEM) in 3
percent glutaraldehyde in phosphate buffer (pH
7.2, 330 mOsm.). For TEM, pieces from half
of each cornea were flat-embedded in a low-
viscosity epoxy resin. Thin sections were cut
with a diamond knife on an LKB ultramicro-
tome (LKB Instruments, Inc., Rockville, Md.)
and viewed in an RCA-EMU-4C transmission
electron microscope. For SEM, hot acetone
was used to wash the resin from the endothelial sur-
face of the other half of each cornea before
polymerization.9 After polymerization, the speci-
mens were sputter-coated with carbon, gold, and
palladium and were viewed with an AMR-1000
scanning electron microscope.

Results

Perfusion of rabbit corneal endothelial
cells with 0.0001 or 0.0005 percent thi-
merosal in GBR caused no significant in-
Effect of thimerosal on corneal endothelium

An increase in the concentration of thimerosal in GBR to 0.001 or 0.005 percent resulted in increases in corneal thickness after 2 hours, which were proportional to the concentration of thimerosal (Fig. 1). Perfusion with 0.01 or 0.1 percent thimerosal resulted in a rapid and immediate increase in corneal thickness.

The endothelium of corneas perfused for 5 hours with GBR alone had minimal ultrastructural changes. SEM revealed that the endothelial cells maintained a continuous flat layer and a normal mosaic-like pattern (Fig. 2, A). TEM revealed that the organelles, junctions, and membranes were normal, although there was some clarification of the cytoplasm near Descemet's membrane (Fig. 2, B). Likewise, perfusion with 0.0001 percent thimerosal in GBR for 5 hours resulted in minimal ultrastructural damage to the endothelial cells as observed by SEM (Fig. 3, A) and TEM (Fig. 3, B).

When rabbit corneas were perfused with 0.0005 percent thimerosal in GBR for 5 hours, SEM revealed that the endothelial cells maintained a mosaic-like pattern, although they appeared swollen and flaps or cytoplasmic extensions had formed around some of the cell borders, indicating the start of junctional breakdown (Fig. 4, A). TEM of these cells showed swelling of the cytoplasm, dilation of the endoplasmic reticulum and nuclear envelope, condensation of the mitochondria, and vacuolization of the apical cytoplasm (Fig. 4, B).

In corneas perfused with 0.001 percent thimerosal and fixed before the cornea began to increase in thickness, the intercellular junctions between the cells were beginning to open up, resulting in long junctional flaps curling upon the cell sur-

Fig. 1. Changes in corneal thickness with time during perfusion of the endothelium of rabbit corneas with various concentrations of thimerosal in glutathione bicarbonate Ringer's solution (GBR). N = number of corneas.

Fig. 2. A, SEM of the endothelium of rabbit cornea perfused with GBR for 5 hours. The mosaic-like pattern is normal. (x1,000.) B, TEM of the endothelium of same cornea. Except for some swelling of the basal cytoplasm, normal ultrastructure is maintained. (x18,000.) (Photographs reduced 60%.)

Fig. 3. A, SEM of endothelium of rabbit cornea perfused with 0.001 percent thimerosal in GBR for 5 hours. The mosaic-like pattern is normal. (x1,000.) B, TEM of the same cornea. Swelling of cytoplasm, dilated endoplasmic reticulum, and condensation of mitochondria are observed. (x18,000.) (Photographs reduced 60%.)

Fig. 4. A, SEM of the endothelium of rabbit cornea perfused with 0.01 percent thimerosal in GBR for 5 hours. Mosaic-like pattern is observed, although some swelling of cytoplasm can be seen. (x1,000.) B, TEM of the same cornea. Swelling of cytoplasm and vacuolization of apical cytoplasm can be seen. (x18,000.) (Photographs reduced 60%.)

Downloaded from iovs.arvojournals.org on 02/11/2019

Fig. 3. A, SEM of endothelium of rabbit cornea perfused with 0.0001 percent thimerosal for 5 hours. The cells are intact, and the normal mosaic-like pattern is maintained. (×1,000.) B, TEM of endothelium of same cornea. Some clarification of basal cytoplasm is present, but organelles, junctions, and membranes appear normal. (×12,000.) (Photographs reduced 60%.)

Fig. 4. A, SEM of endothelium of rabbit cornea perfused with 0.0005 percent thimerosal. The cells appear swollen, and the junctions appear altered. (×1,000.) B, TEM of same cornea. Mitochondria appear condensed, cytoplasmic vacuoles are present, and the junction between the cells is straight with a cytoplasmic flap at the apical end. (×9,000.) (Photographs reduced 60%.)

Fig. 5. In some cases thimerosal was removed at this point and perfusion continued with GBR alone. Corneal thickness stabilized, and normal ultrastructure of the endothelium was restored. In corneas that were perfused with thimerosal until corneal thickness was rapidly increasing, the endothelial cells appeared to be separated, and the posterior surface of the cells appeared rough by SEM (Fig. 6, A). The cells were undergoing necrotic changes as seen by TEM (Fig. 6, B).

Corneas perfused with 0.01 or 0.1 percent thimerosal in GBR had evidence of cellular damage within 1 hour. SEM showed that the endothelial cells had separated, exposing large areas of Descemet's membrane (Fig. 7, A), and TEM revealed that the cells were necrotic (Fig. 7, B). Distortion of the nucleus, clumping of the nuclear chromatin, disruption of the outer plasma membrane, fragmentation of the intracellular membranes, high amplitude mitochondrial swelling, and extensive cytoplasmic vacuolization were present (Fig. 7, B).

For comparative purposes, six human corneas were perfused with either 0.0001 or 0.001 percent thimerosal in GBR for a period of 4 hours. The paired control corneas were perfused with GBR alone for the same length of time. Perfusion with GBR alone or 0.0001 percent thimerosal in GBR did not cause corneal swelling (Fig.
Fig. 5. A, SEM of rabbit endothelium perfused with 0.001 percent thimerosal and fixed before an increase in corneal thickness had occurred. The junctions between the cells are beginning to break down as evidenced by the appearance of many junctional flaps. (×1,000.) B, Endothelial cells with junctional flap in same cornea. (×14,000.) (Photographs reduced 60%.)

8), and the appearance of the endothelium was similar in GBR control and 0.0001 percent experimental corneas (Figs. 9 and 10). In all cases, the normal flat layer of polygon-shaped endothelial cells was present, and the organelles, junctions, and membranes were well maintained.

The human corneas perfused with 0.001 percent thimerosal in GBR increased in thickness after 2 hours (Fig. 8). Following 4 hours of perfusion, the endothelial cell layer remained intact, although individual cells appeared to be swollen and pits were present between cells (Fig. 11, A). TEM examination of the corneas showed cytoplasmic and nuclear swelling, dilation of the intercellular space, cytoplasmic vacuolization, condensation of the mitochondria, and dilation of the endoplasmic reticulum (Fig. 11, B).

Discussion

Widespread use of the organic mercurial-containing preservative thimerosal as an antibacterial agent in many ophthalmic solutions and its possible use in corneal preservation solutions warranted an investigation into the possible cytotoxic effects of this drug on the functional and ultrastructural integrity of the corneal endothelium. Approximately 20 percent of all commercially prepared ophthalmic solu-
Fig. 7. A, SEM of endothelium of rabbit cornea perfused with 0.01 percent thimerosal for 1 hour. The cells appear to have separated, exposing large areas of Descemet's membrane. (x1,000.) B, Portion of a necrotic endothelial cell in same cornea. The nuclear chromatin is clumped, the membranes are discontinuous, and the mitochondria are severely swollen. (x7,000.) (Photographs reduced 60%.)

Reactions are preserved with one of three organic mercurials (i.e., phenylmercuric nitrate, phenylmercuric acetate, or thimerosal [ethylmercurithiobenzoate]). The recommended concentration for mercurials in ophthalmic drugs varies from 0.01 to 0.001 percent. Topical application of 2 percent thimerosal does not damage corneal endothelium in the rabbit. The present investigation, however, indicates that direct exposure of corneal endothelial cells to concentrations of thimerosal that are commonly used in ophthalmic drugs results in functional and structural damage to the endothelium if prolonged. Previous reports have shown that organic mercurials react with membrane sulfhydryl groups, resulting in increases in membrane permeability and alterations of membrane transport systems. For example, one such compound, para-chloromercuribenzenesulfonate (PCMBS), has been used to study the role of sulfhydryl groups in the membranes of red blood cells, isolated kidney tubules, and recently by us in corneal endothelium. These studies have established that normal membrane permeability depends upon the presence of native sulfhydryl groups, and PCMBS exerts its effect by forming mercaptide bonds with the cell membrane sulfhydryl groups. This results in progressive ultrastructural changes from the normal (or steady) state through the sublethal (or reversible) state to a necrotic (or irreversible) state.

A similar progressive pattern of ultrastructural changes was observed following perfusion of increasing concentrations of thimerosal to the corneal endothelium, and this suggests that the mechanism for production of toxic changes may be similar. If so, we can postulate that the sulfhydryl groups of the endothelial cell membranes

Fig. 8. Changes in corneal thickness with time in human corneas perfused with 0.001 and 0.0001 percent thimerosal or GBR alone. N = number of pairs of corneas.
Effect of thimerosal on corneal endothelium

were inhibited by the formation of mercaptide bonds with thimerosal. This would cause an increase in permeability of the cell membrane, resulting in a loss of potassium from the cell and an influx of sodium and water into the cytoplasm and cisternae of the endoplasmic reticulum. This in turn would produce swelling of the cytoplasm, dilation of the endoplasmic reticulum and nuclear envelope, and formation of many cytoplasmic vacuoles (Fig. 4, B). Condensation of the mitochondria (Fig. 4, B) would also occur because of shrinkage of the mitochondrial inner compartments due to the loss of ions and water. The respiratory chain, however, is known to be intact in condensed mitochondria, and all of these changes were reversible when the injurious stimulus (thimerosal) was removed.

When the endothelial cells were exposed to thimerosal for a longer period of time or in a higher concentration, the ionic imbalance apparently continued to occur, resulting in osmotic lysis, cellular death, and necrosis. Such changes could be seen after prolonged perfusion with 0.001 percent thimerosal (Fig. 6) and after short times of perfusion with 0.01 and 0.1 percent thimerosal (Fig. 7). TEM of these corneas displayed extensive damage of the endothelial cells, including disruption of the outer plasma membrane, fragmentation of the intracellular membranes, nuclear distortion and clumping of the chromatin, increased cytoplasmic swelling and vacuolization, and high-amplitude mitochondrial swelling. These changes were irreversible.

In summary, although thimerosal has generally been accepted as a safe preserv-
Fig. 11. A, SEM of human endothelium perfused with 0.001 percent thimerosal for 4 hours. Cells appear swollen, and pits are present between cells. (x1,000.) B, Endothelial cells in same cornea have swollen nuclei and cytoplasm, focal dilation of intercellular space, condensation of mitochondria, and minimal swelling of rough endoplasmic reticulum. (x9,000.) (Photographs reduced 60%.)

tive agent in topical ophthalmic solutions, the data presented in this study indicate that thimerosal, even in the low concentrations (0.001 and 0.005 percent) that are commonly used in commercial preparations, can cause structural and functional damage upon prolonged direct exposure to the endothelium. It is therefore concluded that thimerosal solutions containing thimerosal should not be used during intraocular surgery or in storage solutions for corneal preservation.

REFERENCES