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

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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 endothelial 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.

Cytotoxicity of preservative agents used in ophthalmic drugs and irrigating solutions is being investigated because of the possible deleterious effects of these agents on the corneal endothelium. Several preservatives (e.g., sodium bisulfite, methylparaben, propylparaben, and benzyl alcohol) have been shown to cause changes in corneal thickness and endothelial morphology during in vitro perfusion studies.1-3 Topical application of benzalkonium chloride results in marked cytotoxic effects which may be due to breakdown of the anatomical barrier in the outer layers of the corneal epithelium.4-5 No toxic effects have been observed following topical application of solutions containing thimerosal, even at concentrations 100 times higher than those required for bactericidal effect.1-3 Thimerosal is stable at slightly alkaline or neutral pH and exerts its antimicrobial effect over a wide temperature range (4° to 38° C.). Thimerosal may therefore be the antimicrobial preservative of choice for intracocular drugs and irrigating solutions and for short-term corneal preservation in M-K medium.1

The purpose of this investigation was to compare the toxic effects of various concentrations 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 microscope 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 ultramicrotome (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 surface of the other half of each cornea before polymerization.9 After polymerization, the specimens 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 thimerosal in GBR caused no significant in-

Table I. Concentrations of thimerosal perfused to rabbit corneal endothelium

<table>
<thead>
<tr>
<th>gm./100 ml.</th>
<th>Molar</th>
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<tbody>
<tr>
<td>0.0001</td>
<td>2.47 x 10⁻³</td>
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<tr>
<td>0.0005</td>
<td>2.47 x 10⁻³</td>
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<tr>
<td>0.001</td>
<td>1.34 x 10⁻³</td>
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<tr>
<td>0.005</td>
<td>2.47 x 10⁻³</td>
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<tr>
<td>0.01</td>
<td>1.34 x 10⁻³</td>
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<tr>
<td>0.05</td>
<td>1.34 x 10⁻³</td>
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<tr>
<td>0.1</td>
<td>2.47 x 10⁻³</td>
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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. 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%.)

face (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.
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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. (x1,000.) B, Endothelial cells with junctional flap in same cornea. (x14,000.) (Photographs reduced 60%.)

Fig. 6. A, SEM of rabbit endothelium perfused with 0.001 percent thimerosal for 4 hours. Corneal thickness was increasing rapidly at time of fixation. The endothelial cells appear to be separating, and their posterior surface is no longer smooth. (x1,000.) B, Endothelial cells in same cornea have discontinuities of cellular membranes, high-amplitude mitochondrial swelling, dilation of the cisternae of the rough endoplasmic reticulum, and distortion of the nucleus. (x7,000.) (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 vacuo-

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-
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 sulf-hydryl 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 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.

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. (xl,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%.)

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.
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Fig. 9. A, Human endothelium perfused with GBR for 4 hours. Normal mosaic-like cellular pattern is preserved. (×1,000.) B, TEM of endothelial cells in same cornea. Normal ultrastructure is maintained. (×7,000.) (Photographs reduced 60%.)

Fig. 10. A, SEM of human endothelium perfused with 0.0001 percent thimerosal for 4 hours. Mosaic-like pattern is normal. (×1,000.) B, Normal-appearing endothelial cell in same cornea. (×7,000.) (Photographs reduced 60%.)

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 preserva-

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. (×1,000.) 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. (×8,000.) (Photographs reduced 60%.)

Thimerosal is a preservative 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