Subconjunctival Injections

Preservative-Related Changes in the Corneal Endothelium

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The morphologic effects on rabbit corneal endothelium of several common ophthalmic vehicle constituents were examined following subconjunctival administration. Profound dose-dependent changes consisting of intercellular vacuolization and thickening of the endothelial layer were noted within 1 day following administration of solutions that contained sodium bisulfite or methylparaben and propylparaben. These changes persisted for at least 5 days except in those eyes treated with the lowest concentration of sodium bisulfite. In contrast, administration of sodium citrate and creatinine or unpreserved normal saline resulted in only minimal effects. These changes are of concern because these agents are present in many preparations used to treat a wide variety of eye diseases. Invest Ophthalmol Vis Sci 27:525-531, 1986

Materials and Methods

Forty-eight New Zealand albino rabbits of both sexes (weighing 2.5 to 3.5 kg) were treated with four solutions at three concentrations. The highest concentrations of the solutions were (1) sodium bisulfite 0.2%, (2) methylparaben 0.3% and propylparaben 0.04%, (3) creatinine 0.16% and sodium citrate 2.0%, and (4) a combination of the first three solutions. We refer to these four solutions as high concentration solutions. Each of these solutions was diluted two-fold (intermediate concentration) and eight-fold (low concentration) in normal saline. The twelve resulting preparations were then administered by subconjunctival injection (0.18 ml) to one eye of three animals to acquire a measure of a dose-response relationship. The intermediate concentration of solution four is the same as the vehicle of Decadron Phosphate (dexamethasone sodium phosphate; Merck, Sharpe, and Dohme, West Point, PA) and is subsequently called “vehicle.” Eyes treated with unpreserved saline (0.23%, 0.9%, or 1.8%) or which were not treated served as controls.

Subconjunctival injections were placed in the superotemporal quadrant following a drop of 0.5% proparacaine (Ophthaine®, Squibb; Princeton, NJ), and a small bleb was raised. For each solution, eyes of two animals were enucleated after 1 day and those of one animal after 5 days.

Clinical Evaluation

Prior to enucleation, corneal clarity was assessed by a slit-lamp examination and specular microscopy. Specular microscopy was performed with a wide-field specular microscope, using a 20× dipping-cone objective lens. Five fields of each cornea were examined and photographed. The photographic prints were evaluated in a masked fashion. In an additional ten rabbits, corneal thickness was assessed with a CS-1000 pachymeter (Storz; St. Louis, MO). One eye in each of two rabbits was treated for each of the four solutions and unpreserved saline at high concentrations. Measurements were obtained 30 min, 4 hr, 24 hr, and 72 hr after subconjunctival administration in both treated and

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untreated eyes. We evaluated the corneal thickness in the treated eye by subtracting the thickness at baseline from the thickness at each time interval.

Specimen Preparation

Eyes were enucleated following an intravenous injection of ketamine (30 mg/kg) and xylazine (6 mg/kg). The anterior segment containing cornea, trabecular meshwork, iris, and ciliary body was excised 2 mm from the limbus and immediately immersed in fixative consisting of 2% glutaraldehyde, 1% paraformaldehyde, with 0.2 M sodium cacodylate buffer (final concentration 0.06 M) and 0.2% calcium chloride at pH 7.3 for 3-4 hours. Following the primary fixation, the specimens were washed in 0.068 M sodium cacodylate buffer (pH 7.3), and post-fixed in 1% osmium tetroxide in sodium veronal acetate buffer (final concentration 0.06 M and pH 7.3), with the addition of potassium ferri cyanide (reduced osmium method) for 2 hr. The specimens were then washed in 0.06 M sodium veronal acetate buffer, en bloc stained with 0.5% uranyl acetate, dehydrated in graded acetone 50–100% and propylene oxide, embedded in Araldite 502 applying long-term infiltration, and polymerized.

One-micron sections were cut and stained with basic fuchsin and methylene blue. A photomicroscope (Carl Zeiss, Inc.; Oberkochen; West Germany) was used to examine and photograph all of the specimens. Ultrathin sections of the same areas were sectioned on a Sorvall (Newtown, Connecticut) MT-2B ultramicrotome, stained with 2% uranyl acetate and lead citrate. The sections were examined and photographed using a JEOL (Tokyo, Japan) 100 C transmission electron microscope.

Morphometric Analysis

At one day, in one treated eye for each solution, three sections were taken at random through the central 6 mm of corneal endothelium by a masked individual. A linear length of 230 μm was photographed in each of these at ×1040 magnification. Another masked individual measured the vacuolization within the endothelial layer by characterizing the vacuolized area (mm²)/100 mm² of endothelium with a Graf-Pen GP6-Model 40 digitizer (Science Accessories Corp.; Southport, Connecticut). The vacuolized areas for each specimen were pooled and statistically compared.

Osmolality and pH

The aqueous humor of 12 additional rabbits was obtained by paracentesis with a #27 needle on a tuberculin syringe to measure osmolality with an Advanced Digimatic osmometer (Advanced Instruments Inc.; Needham, Massachusetts) and pH with a digital pH meter Model 3500 (Beckman; Palo Alto, CA). Samples were obtained at 30 min, 2 hr, 4 hr, and 24 hr after subconjunctival administration of each of the four solutions at high concentration.

Results

Clinical Evaluation

Within 1 day following subconjunctival injection, dose-dependent morphologic changes of the central corneal endothelium were found in the rabbits that received either methylparaben and propylparaben (solution 2) or the "vehicle" (solution 4). Less marked changes were observed in the eyes treated with sodium bisulfite (solution 1). The eyes treated with either creatinine and sodium citrate (solution 3) or unpreserved saline were essentially unchanged, and the latter were identical to untreated rabbit eyes. We noted no differences between treated and control eyes with slit-lamp biomicroscopy. Masked evaluation of the specular photographs revealed no distinct differences between treated and control eyes. We have the impression, however, that there was blurring of the cell borders in treated eyes during the first 5 days, especially those to which the highest concentrations of bisulfite and parabens were administered (Fig. 1). This was not seen 1
month after treatment. No clear change in corneal thickness was detected.

**Light Microscopy**

Only a few, tiny vacuoles were observed within the homogenous cytoplasm of the central corneal endothelium of the untreated control eyes (Fig. 2A). In eyes treated with creatinine or sodium citrate (solution 3) this normal appearance was preserved except for the presence of a few small vacuoles (Fig. 2B). In contrast, the rabbits with the “vehicle” showed profound changes (Figure 2C). These changes consisted of vacuoles in the central corneal endothelium, which frequently were as large or larger than the endothelial nucleus itself. In addition, the endothelial monolayer acquired an irregular configuration and a moderate increase in thickness. The rabbits given a subconjunctival injection of unpreserved saline showed a normal endothelium that was identical to the untreated control.

The changes shown by the “vehicle” could be reproduced when the eyes were treated with either the bisulfite (solution 1) or the parabens (solution 2) alone. Figure 2D shows a representative portion of the central corneal endothelium in a rabbit treated with the lowest concentration of sodium bisulfite (0.025%) and examined 1 day after treatment. This endothelium was only lightly affected as there were more vacuoles in these endothelial cells than in the normal endothelium as shown in Figure 2A. The size of the vacuoles increased when the dosage of the bisulfite was increased four-fold (0.1%) as shown in Figure 2E. A further increase in the concentration of the sodium bisulfite (0.2%) produced even a greater increase in the vacuolar size as well as an increase in the thickness of the endothelial cells (Fig. 2F).

A more dramatic change in the appearance of the endothelium could be demonstrated when the parabens were given alone (solution 2). Figure 2G shows the corneal endothelium in a rabbit treated with the lowest concentration of the parabens (0.04% methylparaben and 0.005% propylparaben). The endothelium showed only a few changes that consisted mainly of the presence of an increased number of small vacuoles. When the
concentration of the parabens was increased four-fold, there was a dramatic increase in the size of the vacuoles and in the disruption of the endothelial monolayer (Fig. 2H). A further doubling of the concentration produced an additional increase in the vacuolar size and disruption of the monolayer as shown in Figure 2I. There were no differences between the appearance of the corneal endothelium after 1 day and 5 days, except in the corneas which had received the lowest concentration of sodium bisulfite alone (solution 1). In these corneas, there was a resolution of the vacuolization so that the endothelium was not even lightly affected and looked normal.

Electron Microscopy

The changes described above were examined by electron microscopy to investigate further the profound effects that these substances had on the normal appearance of the corneal endothelium. The corneal endothelium of the untreated control group had a homogenous cytoplasm and a narrow intercellular space as well as two rather parallel surfaces on its inner and outer borders (Fig. 3A). Except for a few cytoplasmic vacuoles, this was also demonstrated (Fig. 3B) in the eyes treated with the intermediate concentrations of sodium citrate and creatinine (solution 3). Following the injection of the “vehicle” (solution 4) at the intermediate concentration, a marked change in the appearance and size of the endothelium was observed. Further, the intercellular space was also widened to form an intercellular vacuole (Figure 3C). The cytoplasm showed some disrupted mitochondria, a few cytoplasmic vacuoles, and multi-vesicular bodies. Following the injection of sodium bisulfite (solution 1) at

Fig. 3. Transmission electron microscopic changes in central rabbit corneal endothelium after subconjunctival injection of (a) unpreserved saline (or untreated control); (b) creatinine or sodium citrate; (c) “vehicle”; (d) sodium bisulfite, and (e) parabens (methyl- and propyl-) at high concentrations (X12,500).
Table 1. Vacuoles (mm²/100 mm²) in the central corneal endothelium of rabbits treated with subconjunctival injection of a variety of solutions (n = 3, for all solutions) at 1 day. Statistical comparisons with control were done using paired two-tailed t-tests and a P-value of 0.05 was considered significant.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Percent vacuolized area (Mean ± S.D.)</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Bisulfite</td>
<td>Low: 7.27 ± 3.31</td>
<td>N.S.*</td>
</tr>
<tr>
<td></td>
<td>Intermediate: 18.87 ± 11.33</td>
<td>N.S.*</td>
</tr>
<tr>
<td></td>
<td>High: 40.77 ± 0.06</td>
<td>*P &lt; 0.001</td>
</tr>
<tr>
<td>Parabens</td>
<td>Low: 1.86 ± 0.43</td>
<td>*P &lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>Intermediate: 24.74 ± 6.99</td>
<td>*P &lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>High: 38.32 ± 1.34</td>
<td>*P &lt; 0.001</td>
</tr>
<tr>
<td>Creatinine and Citrate</td>
<td>6.17 ± 1.45</td>
<td>N.S.*</td>
</tr>
<tr>
<td>Vehicle</td>
<td>44.60 ± 2.63</td>
<td>*P &lt; 0.01</td>
</tr>
<tr>
<td>Control</td>
<td>0.52 ± 0.15</td>
<td>—</td>
</tr>
</tbody>
</table>

* No statistical significance.

the intermediate concentration, the endothelium also formed large intercellular vacuoles (Fig. 3D). Intracellular vacuoles in disrupted mitochondria were also observed. A greater disruption of the endothelium was produced by the parabens (solution 2) as there was formation of larger-sized intercellular vacuoles and multi-vesicular bodies (Fig. 3E). The rest of the cytoplasm and nucleus appeared to be normal in appearance.

Morphometric Analysis

We found a clear dose-dependent relationship when the degree of vacuolization at 1 day and the administered preservative concentration were compared by morphometric methods. The percent of endothelial area occupied by vacuoles for each tested solution is shown in Table 1. In the case of the sodium bisulfite (solution 1) and parabens (solution 2) groups, the area of vacuolization increased in proportion to the administered dose. At the lowest concentrations of bisulfite, the vacuolization was not significantly different from the control group. In the eyes treated with the parabens, a significant difference was noted even at the lowest concentration (P < 0.05). The vacuolization of the eyes treated with “vehicle” (solution 4), which included the parabens and bisulfites at their intermediate concentrations, was the same as that caused by each of these preservatives separately administered. The eyes exposed to unpreserved normal saline (control) or sodium citrate and creatinine (solution 3) showed a minimal amount of vacuolar formation. The difference between those two groups was not statistically significant.

Osmolality and pH

The osmolality was between 233 mOsm and 306 mOsm in the administered solutions and between 292 mOsm and 307 mOsm in the aqueous samples (Table 2). These values are within the range of those reported to leave corneal endothelium unchanged, even after in vitro perfusion. The pH was 7.06-8.46 in the administered solutions and 7.80-7.95 in the aqueous samples (Table 3). The pH values are also within the range of those reported to maintain the functional and ultrastructural integrity of the rabbit corneal endothelium, even after in vitro perfusion. Furthermore, there was no relationship between the extent of the observed changes in the corneal endothelium and the osmolality or pH of the administered solutions or resulting aqueous samples.

Table 2. Osmolality (mOsm/kg) of administered solution and resulting aqueous samples at various time intervals. Osmolality of untreated rabbit aqueous was 295 mOsm/kg.

<table>
<thead>
<tr>
<th>Solution</th>
<th>30 min</th>
<th>2 hr</th>
<th>4 hr</th>
<th>24 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium bisulfite</td>
<td>288</td>
<td>302</td>
<td>299</td>
<td>—</td>
</tr>
<tr>
<td>Parabens</td>
<td>295</td>
<td>305</td>
<td>303</td>
<td>—</td>
</tr>
<tr>
<td>Creatinine and citrate</td>
<td>306</td>
<td>294</td>
<td>302</td>
<td>—</td>
</tr>
<tr>
<td>Vehicle</td>
<td>233</td>
<td>295</td>
<td>294</td>
<td>294</td>
</tr>
<tr>
<td>Normal saline</td>
<td>294</td>
<td>292</td>
<td>307</td>
<td>—</td>
</tr>
</tbody>
</table>

Table 3. pH of administered solution and resulting aqueous samples at various time intervals. pH of untreated rabbit aqueous was 7.4.

<table>
<thead>
<tr>
<th>Solution</th>
<th>30 min</th>
<th>2 hr</th>
<th>4 hr</th>
<th>24 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium bisulfite</td>
<td>7.36</td>
<td>7.90</td>
<td>7.92</td>
<td>—</td>
</tr>
<tr>
<td>Parabens</td>
<td>7.63</td>
<td>7.88</td>
<td>7.86</td>
<td>—</td>
</tr>
<tr>
<td>Creatinine and citrate</td>
<td>8.34</td>
<td>7.87</td>
<td>7.95</td>
<td>—</td>
</tr>
<tr>
<td>Vehicle</td>
<td>7.21</td>
<td>7.83</td>
<td>7.88</td>
<td>—</td>
</tr>
<tr>
<td>Normal saline</td>
<td>7.06</td>
<td>7.91</td>
<td>7.80</td>
<td>—</td>
</tr>
</tbody>
</table>
Discussion

This investigation confirmed previous reports that some constituents of the vehicle may be toxic to the rabbit corneal endothelium.\textsuperscript{3,4,7,8} We demonstrated that this toxicity can be produced by several common vehicle constituents in a dose-dependent manner. While solutions that included sodium bisulfite or methylparaben and propylparaben caused profound morphologic changes in the central corneal endothelium, the clinical significance of these changes, as determined by measurement of corneal thickness, was not readily apparent. Since corneal thickness increases only when the pump and barrier functions of the endothelium have been substantially compromised, it is possible that the functional reserve of the endothelium enabled it to maintain normal corneal thickness despite the morphologic changes.

Preservative-related changes in the corneal endothelium are of concern because these agents are present in many preparations used to treat a wide variety of ophthalmic diseases. Earlier studies examined the ocular toxicity of thimerosal,\textsuperscript{7,8} chlorbutanol,\textsuperscript{8} and sodium bisulfite.\textsuperscript{3,4} Thimerosal was highly toxic to the rabbit corneal endothelium when administered by in vitro perfusion,\textsuperscript{7} but not after topical administration.\textsuperscript{8} Chlorbutanol also did not seem to have any effect after being administered topically.\textsuperscript{8} In contrast, topical administration of benzalkonium chloride resulted in pronounced cytotoxicity after only two drops of 0.1% solution (8). Sodium bisulfite has been the most widely investigated preservative. It was highly toxic to the corneal endothelium when administered by intracameral injection\textsuperscript{9} or after in vitro perfusion in rabbits.\textsuperscript{7} In contrast, the corneal endothelium of cats, which like human cornea and unlike that of rabbits has a limited regenerative capacity, was not damaged after intracameral injection of sodium bisulfite as determined by specular microscopy.\textsuperscript{9} Although the parabens are associated with an allergic contact dermatitis\textsuperscript{10} and may cause lid swelling, ocular toxicity of methylparaben and propylparaben have not been previously reported to our knowledge. However, one investigation had data demonstrating that the addition of these preservatives to certain antibiotics caused abnormal corneal swelling in vitro.\textsuperscript{11}

In our investigation, the profound effects on the corneal endothelium were most likely mediated by the direct absorption of the preservatives into the eye through the cornea or sclera after subconjunctival administration. Hence, our results reaffirm that even topical administration of drugs can cause ultrastructural changes in the rabbit cornea.\textsuperscript{8,12} For example, topical administration of phenylnephrine was observed to be cytotoxic to corneal endothelium with resulting intracellular vacuolization.\textsuperscript{12} It has also been suggested that topical administration of sodium bisulfite may contribute to the decreased endothelial density found after chronic epinephrine (Glaucon\textsuperscript{®}; Alcon Labs, Inc., Ft. Worth, TX) administration in glaucoma patients.\textsuperscript{13}

We observed changes in the central endothelial layer within a short period of time (1 day) following subconjunctival injection. Cells were altered with the formation of intercellular vacuoles. These changes were reversible within 5 days in those eyes which had received the lowest concentrations of sodium bisulfite. After 30 days, even the pronounced vacuolization of eyes treated with the highest concentration of vehicle had almost completely reversed. Contralateral eyes of rabbits in whom large concentrations of drug were administered were similarly affected, most likely because of absorption into the intracommunicating artery of the rabbit or substantial systemic absorption. At the lowest concentrations, the contralateral eye was not affected or only minimally changed.

Certain drug properties are known to affect the corneal endothelium. For example, the corneal endothelium can tolerate a wide range of solution osmolalities (200–400 mOsm) without marked cell breakdown, provided essential ions are present in the perfusion medium or vehicle.\textsuperscript{5} Cell structure and function can also be affected by the ionic buffer capacity, substrate composition, and pH.\textsuperscript{6} The length of time that the substance remains in the eye and its concentration are also important. This is determined by the formulation of the drug as well as certain characteristics of the eye, such as the rate of aqueous formation (which determines substance turnover in the anterior chamber), the buffering capacity of the aqueous or vitreous, and their initial pH. Rabbit corneal endothelium may be more susceptible to the toxic effects of a drug than human corneal endothelium.

Although the observed effects did not appear related to the osmolality and pH of the administered solutions, it is possible that local changes in these values could result in such deleterious changes. Hence, if the preservatives or their metabolites localized on the cell surface, they could possibly cause an osmotic gradient with subsequent vacuolization. This hypothesis could be tested by using high performance liquid chromatography to examine the membranes of endothelial cells cultured in media to which the preservatives were added. Morphologic changes could also be the result of cytotoxicity. In this regard, topically administered phenylnephrine causes intracellular vacuolization and loss of intercellular adhesion in monolayer cultures of bovine central endothelium.\textsuperscript{14} This in vivo effect is transient, probably because of drug washout by aqueous flow and/or metabolism.\textsuperscript{14,15} It is also possible that the observed changes may be related to a disturbance of
the normal adhesion of the endothelial cells to themselves and/or Descemet's membrane since intracellular vacuolization was not observed after the preservatives were introduced to cultures of bovine corneal endothelial cells at concentrations similar to those employed in the current investigation (unpublished data: Jorge Alvarado and Dennis Fuji). Such effects could be mediated by alterations in the normal relationships between the extracellular matrix and endothelial cell cytoskeleton.

The implications these data have for ocular drug administration to humans and other species must be evaluated further. They should only be applied with caution since multiple factors undoubtedly contribute to such a response. Continued evaluation of the morphology of normal and stressed corneal endothelium will clarify the relationship between these characteristics and endothelial functions.

Key words: preservatives, sodium bisulfite, parabens, corneal endothelium, toxicity

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References