The Effect of Sodium Hyaluronate on the Corneal Epithelium

An Ultrastructural Study

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The effect of sodium hyaluronate (NaHa) 1% and 0.1% was studied on 14-day chick embryo corneal epithelium by scanning electron microscopy. It was found that NaHa 1% or 0.1% had no toxic effects on the chick corneal epithelium and the normal architecture of the cells and the morphology of the microvilli was well preserved. A combination of NaHa 0.1% and benzalkonium chloride (BAK) 0.01% reduced the toxic effect of BAK on the surface corneal epithelium. NaHa 0.1% provided a better protection of the corneal epithelium against dryness than hydroxyethylcellulose (HEC) 0.1% or phosphate buffer saline (PBS). Invest Ophthalmol Vis Sci 29:194-199, 1988

The precorneal "tear film" is a three-laminar-fluid complex 5-10 μm in thickness. The three components are: a lipid layer (about 0.1 μm) on the outer side facing the atmosphere, an intermediate watery layer (7 μm) and the innermost mucin layer (0.02-0.05 μm). Recent work indicates that the mucin layer is 20-fold thicker than thought before and varies from 0.4 to 1.0 μm.

A defect in one or more of the tear fluid components causes the syndrome routinely called "dry eye." This disorder is expressed in chronic ocular discomfort, burning, itching, photosensitivity and grittiness.

Of all treatment modalities available for the "dry eye," the application of "artificial tears" is still the most common. These tear supplement solutions contain hydrophilic polymers, which lubricate the eye during blinking and thus prevent the eye from drying when open.

Because of the relatively short retention time, the tear substitutes have to be used every hour or more frequently to be effective. Preservatives are usually added to ophthalmic solutions to prevent contamination and to achieve a longer shelf life. Benzalkonium chloride (BAK) is one of the common preservatives which provides stability and antimicrobial effectiveness to the solution to which it is added. However, the powerful cationic detergent action of BAK which destroys bacteria by ionic attraction makes it most harmful to the lipid layer and to the membranes of the corneal epithelium. It was shown that 0.01% solution of BAK reduces the tear breakup time (B.U.T.) by half. It was also found that in rabbits the use of BAK 0.01% caused loss of microvilli, membrane disruption, desquamation and death of superficial cells.

Healon® (Pharmacia, Uppsala, Sweden), regularly used in intraocular surgery, is a high molecular weight, viscoelastic noninflammatory preparation. It creates and maintains a deep anterior chamber, separates membranes, maneuvers tissues and protects intraocular tissues especially the corneal endothelium. Recently the clinical use of NaHa was expanded to extraocular treatment. Several investigators have treated dry eye patients with NaHa 0.1% solution, demonstrating subjective improvements in patient comfort and decreasing symptoms. It was also found that the stability of the tear film was significantly increased.

The effects of NaHa on the ultrastructure of the corneal epithelium are as yet largely unknown. The purposes of this study were: (1) to determine whether NaHa has a toxic effect on the corneal epithelium; (2) to examine if NaHa in combination with BAK reduces the toxic effects of BAK on the corneal epithelium; and (3) to determine if NaHa has any advantage over HEC or PBS in providing protection to the corneal epithelium.

Eyes of 14-day-old chick embryos served as the experimental model system throughout this study.

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Fig. 1. Scanning electron micrograph of surface corneal epithelium from 14-day-old chick embryo. (A) Low magnification showing densely packed hexagonal cells covered with microvilli. Light cells (L) differ from dark cells (D). Scale bar = 10 μm. (B) The dense and long microvilli covering the corneal epithelium are clearly seen at high magnification. Scale bar = 1 μm.

Materials and Methods

General

Fourteen-day-old chick embryos were sacrificed and the eyes were enucleated. Immediately after the various treatments, the eyes were prepared for scanning electron microscopy (SEM) as follows: the whole eyes were fixed with 1% glutaraldehyde in PBS pH 7.4 at room temperature for 1 hr. Then the corneas were excised next to the limbus, additionally fixed for 15 min with 1% glutaraldehyde and postfixed with 1% OsO₄ in PBS for 10 min. The corneas were processed for SEM by dehydration in a series of graded alcohols and critical point drying from CO₂. The preparations were then coated with gold (sputter coating) and were examined with a Jeol (Tokyo, Japan) JSM-35 scanning electron microscope.

Chemicals

Chemicals used were: Healon® (1% sodium hyaluronate) (Pharmacia); Lyteers® (0.1% hydroxy ethyl cellulose, 0.01% Benzalkonium chloride) (Barnes Hind); and Hydroxy ethyl cellulose 0.1% diluted in PBS.

Morphometry

Evaluation of the damage caused by BAK in HEC, (Lyteers) or in NaHa 0.1% was performed on scanning electron micrographs (final magnification ×1000) taken at random from the apical area of treated corneas. Six eyes were used for each treatment and from each eye four micrographs, covering a total area of 36,000 μm² (about 120 cells), were screened. Cells were divided into three categories according to the morphology of the cell surface. The number of epithelial cells in each category was counted on all micrographs of a given treatment and expressed as a percentage of the total number of cells screened for that treatment.

Results

Eyes of 14-day-old chick embryos are relatively large, about 8 mm in diameter. Their corneal epithelium is made of packed hexagonal cells covered with dense microvilli. “Light” cells, covered with more microvilli, are clearly distinguished from “dark” cells (Fig. 1). Thus, the surface of a chick embryo cornea resembles the epithelium of human cornea. However, the human surface cornea cells are less regular and show some variability in shape and size.
Fig. 2. Scanning electron micrograph of a cornea immersed in NaHa 1% for 90 min. Note that the microvilli are well preserved and the cell membrane is intact. Bar = 1 μm.

Fig. 3. Surface epithelium of cornea immersed in Lyteers (HEC 0.1% + BAK 0.01%) for 15 min. Cells with total loss of microvilli (T), cells with reduced number of microvilli (R) and cells with normal morphology (N) are distinguishable. Bar = 10 μm.

Fig. 4. Cornea immersed in 0.1% NaHa + 0.01% BAK for 15 min. Note the well preserved architecture of the cells. Bar = 10 μm.

The first experiment was to determine whether NaHa has toxic effects on the chick embryo ocular surface. Whole eyes were immersed in either a NaHa solution of 1% or 0.1% in PBS for 90 min at room temperature. Despite the long treatment no toxic effects were detected. It was clearly observed that the normal architecture and morphology of the microvilli were well preserved (Fig. 2).

The preservative BAK is known to be toxic when used alone or in combination with the drug it preserves. Therefore, in the second set of experiments it was tested in combination with NaHa. Chick embryo eyes were immersed in a solution made of 0.01% BAK and 0.1% NaHa in PBS for 15 min at room temperature. These treated eyes were compared to eyes immersed in commercial solution of Lyteers (0.1% H.E.C. and 0.01% BAK in PBS). It was found (Fig. 3) that eyes immersed in Lyteers exhibited corneas with many damaged cells. In some cells a severe loss of microvilli was noted, in others large “holes” in the cell membranes were observed. In comparison, corneas treated in the same manner by 0.1% NaHa combined with 0.01% BAK benefited from a reduction in the number of damaged cells and thus from a lessening of the extent of the total damage (Fig. 4). In order to substantiate these findings a morphometric study was carried out. The cells in the treated eyes...
Table 1. Morphometric evaluation of the damage caused to corneal epithelium by benzalkonium chloride in the presence of hydroxy ethyl cellulose or sodium hyaluronate

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of cells screened</th>
<th>Dense microvilli*</th>
<th>Partially damaged microvilli*</th>
<th>Total loss of microvilli*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyteers® (0.1% HEC +0.01% BAK)</td>
<td>717</td>
<td>49</td>
<td>24</td>
<td>27</td>
</tr>
<tr>
<td>0.1% NaHa+0.01% BAK</td>
<td>685</td>
<td>83</td>
<td>14</td>
<td>3</td>
</tr>
</tbody>
</table>

* Expressed as percentage of screened cells.

were divided into three subpopulations according to the severity of the damage to their microvilli caused by BAK (see Materials and Methods). Table 1 summarizes the morphometric studies. It can be noted that about 50% of the cells were damaged following treatment with Lyteers, among which 27% of these were severely affected. In contrast, only 17% of the NaHa + BAK treated cells were damaged and of those only very few totally lost their microvilli. Statistical analysis of cells treated with BAK in the presence of NaHa in comparison to cells treated with BAK in the presence of HEC have clearly shown that the two populations are highly significantly different: X² = 205 for df = 2; P < 10⁻⁹.

An additional set of experiments was carried out to determine whether NaHa 0.1% is better than HEC or PBS in protecting the embryonic corneas from drying. Two drops of each of the tested solutions were instilled on the corneas and then the eyes were placed in a petri dish for 5 min at 37°C. It was found that the damage caused to the epithelial cells by dryness was maximal at the apex of the eyes and more moderate at the periphery of the cornea. Therefore, three areas were examined for each of the treatments: the apex, the mid-periphery and the periphery (Figs. 5-7). In eyes treated with PBS (Fig. 5) severe damage to the corneal epithelium could be observed. Cell borders disappeared and destruction of microvilli in all three areas was obvious. In eyes treated with HEC the damage was severe in the apical area, whereas in the mid-periphery and peripheral areas, cell borders were distinct and the microvilli were longer and denser (Fig. 6). In contrast to PBS or HEC, NaHa protected the cornea effectively against dryness. Even in the apex of the cornea the damage to the cells was moderate whereas in the periphery and mid-periphery (Fig. 7) the cell borders were distinct and the microvilli had relatively normal appearance.

Discussion

During the last few years encouraging reports have appeared indicating the advantages of using NaHa as a tear substitute in the treatment of dry eye patients. In light of these findings, detailed ultrastructural investigation on the effects of NaHa on the corneal epithelium seems to add an important dimension. Such a study is presented here. Chick embryo eyes were found to offer a favorable experimental system for several reasons: chick embryo eyes are relatively large, the corneal epithelium is made of polygonal cells with clear borders, the cells are covered with distinct microvilli and resemble parallel cells in adult mammalian eyes. In addition, large quantities of eyes required for statistical evaluation are readily available, and last but not least the experiments can be carried out conveniently and they are inexpensive.

Fig. 5. A mid-periphery area of surface epithelium from corneas treated by PBS at 37°C for 5 min. Note total change in surface morphology. The surface is wrinkled and curved, cell borders are indistinguishable and the microvilli are very short and sparse. Bar = 10 μm.
It was already shown\textsuperscript{17,18} that NaHa causes no damage to intraocular tissues. The results of the presented study clearly demonstrate that instillation of NaHa (0.1\% or 1\%) on the epithelial cells of chick embryo corneas has no toxic effects. The normal morphology and size of the microvilli are well preserved—no partial or total loss of microvilli, disruption or desquamation of cells were observed. This is in agreement with clinical results\textsuperscript{14} which have clearly shown that treatment of patients with NaHa 0.1\% for a period of 2 years had no adverse effects.

Using the chick embryo experimental system it was proved that BAK is harmful. Lyteers (HEC 0.1\% + 0.01\% BAK) caused damage to the epithelial cells expressed in loss of microvilli and the appearance of large holes in the cell membranes. HEC when used by itself caused no damage. A combination of NaHa 0.1\% with BAK, however, was effective in reducing the toxic effects of BAK. A possible explanation may be that ionic attraction between the positive charge of BAK and the negative charge in NaHa neutralizes the toxic effect caused by the cationic charge of BAK to the corneal epithelium. It is also possible that the BAK small molecules penetrate into the sponge-like domain of NaHa and disperse within it. As a result, their real concentration and chemical activity will be different in combination with NaHa than as a free solution. It has to be emphasized that the reduction of toxic effects by the combination of NaHa and BAK is of value for another reason. NaHa is currently distributed without a preservative. If used as tear drop preparation, in the future NaHa will have to be combined with a preservative in order to be an agent with long shelf life.

The ultrastructural studies performed have shown that NaHa protects the corneal epithelium against dryness better than PBS and HEC. These results are in agreement with clinical tests\textsuperscript{19} in which various protecting solutions were compared during cataract extraction surgery. The protecting effects of NaHa may be attributed to its special sponge-like structure of polysaccharide chains with trapped water. It is assumed that these water molecules are slowly released from the NaHa solution and thus provide a wetting medium to the epithelium, protecting it for relatively longer periods. NaHa behaves as a non-Newtonian fluid with characteristics similar to tear mucose glycoprotein.\textsuperscript{20} It shears thin at high rates and thus permits the advantages of high viscosity in between eye blinking to be added to those of lower viscosity during the blink. The longer adherence of NaHa to the corneal epithelium\textsuperscript{12} results in increased tear stability.

Based on the present study and on recent clinical trials, it seems that NaHa is indeed a potentially useful preparation for treatment of dry eyes. However,
further studies, both morphological and physiological, on its effect on mammalian corneas are required before reaching a final conclusion.

Key words: chick embryo, corneal epithelium, sodium hyaluronate, benzalkonium chloride, hydroxy ethyl cellulose, scanning electron microscope

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References