Toxicity of Natural Tear Substitutes in a Fully Defined Culture Model of Human Corneal Epithelial Cells

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PURPOSE. Serum and saliva have recently been advocated as natural tear substitutes for intractable aqueous-deficient dry eyes, but the effects of these fluids on corneal epithelium have not been well characterized. A laboratory study was performed in a defined test model to compare the toxicity of natural and pharmaceutical tear substitutes and to identify potentially toxic factors in natural tear substitutes, such as amylase, hypotonicity, and variations in preparation.

METHODS. Primary human corneal epithelial cells were cultured with defined keratinocyte serum-free medium. The cells were incubated with hypromellose (hydroxypropylmethylcellulose 0.3%) with and without benzalkonium chloride 0.01%, saliva with differing osmolalities, 100% serum, and 50% serum (1:1 vol/vol with chloramphenicol 0.5%) for varying times and concentrations. Toxicity was examined in four ways. Microvillous density was assessed with scanning electron microscopy. Cell membrane permeability and intracellular esterase activity were analyzed after staining with fluorescent calcein-AM/ethidium homodimer and cellular adenosine triphosphate (ATP) was quantified using a luciferin-luciferase–based assay.

RESULTS. The toxicity ranking of the tear substitutes correlated in all assays. The ATP assay was the most sensitive, followed by ethidium cell permeability, and finally the esterase activity. Preserved hypromellose was more toxic than the unpreserved preparation. Among natural tear substitutes, natural saliva was most toxic. Isotonic saliva and 50% serum were of similar toxicity, and 100% serum was least toxic. Natural tear substitutes were—except for natural saliva—less toxic than unpreserved hypromellose. Hypotonicity, but not amylase, was the major toxic effect associated with saliva. The dilution of serum with chloramphenicol induced toxicity.

CONCLUSIONS. This is the first toxicity study using human primary corneal epithelial cells cultured under fully defined conditions as an in vitro model. Cellular ATP is a sensitive parameter for quantifying toxicity. Isotonic saliva and serum offer greater therapeutic potential for severely aqueous-deficient dry eyes than do pharmaceutical tear substitutes. (Invest Ophthalmol Vis Sci. 2001;42:948–956)

The natural tear film combines excellent protection against desiccation and biomechanical stress, with antimicrobial and nutritional activities for the ocular surface. According to current concepts, aqueous-deficient dry eyes are a result of immune-based inflammation of the ocular surface and cytokine-driven abnormal lacrimal acinar and conjunctival epithelial cell death which finally lead to ocular surface irritation.1,2 Anti-inflammatory management options, such as cyclosporin, are currently being evaluated,3,4 but palliative pharmaceutical tear substitutes remain the most widely used treatment.2 Although mild to moderate aqueous deficiency can be well managed with unpreserved pharmaceutical lubricants, patients with absolute tear deficiency may require additional nutritional substitutes to control the signs and associated symptoms of keratoconjunctivitis sicca. The ideal tear substitute for these would be able to lubricate and nourish the ocular surface and be easily available in an unpreserved, stable preparation.

The biomechanical properties of pharmaceutical tear substitutes have been optimized.5 It is also well established that the addition of preservatives should be avoided in tear substitutes, because they break down the functional barrier and induce morphologic changes in corneal epithelial cell membranes.6–8 The most widely used preservative, benzalkonium chloride (BAC), is retained within epithelial cell membranes for several days. High concentrations of this cationic detergent lead to cell necrosis, whereas concentrations as low as 0.0001% induce growth arrest and apoptotic cell death. Unpreserved artificial tears with slightly hypotonic or physiologic electrolyte composition and biologic buffers improve corneal epithelial barrier function and patient comfort.9,10 However, few attempts have been made to copy the complex nutritional properties of natural tears in pharmaceutical tear substitutes. Fibronectin11 and growth factors such as epidermal growth factor (EGF)12,13 or basic fibroblast growth factor (bFGF)14 are known to accelerate corneal epithelial wound healing through stimulation of proliferation and migration and antiapoptotic effects.15,16 They have been evaluated as single agents in vitro and in vivo, but only vitamin A has been established in routine clinical treatment of dry eyes on a wider basis,17 because of limitations of stability as well as efficacy of single compound preparations.10,18–21 Recently, the use of natural tear substitutes such as autologous serum or submandibular gland saliva have been advocated for the treatment of intractable aqueous-deficient dry eyes.22,23 They are unpreserved, nonallergenic, and provide nutritional factors found in natural tears.24,25 Disadvantages are potential instability and compositional differences compared with natural tears, including higher concentrations of transforming growth factor (TGF)-β in serum and high amylase activity and low osmolality in saliva.

We performed a laboratory study on cultured human corneal epithelial cells to compare the toxicity of natural with pharmaceutical tear substitutes. We also tried to determine the role of factors such as amylase, the hypotonicity and variations in preparation, such as serum dilution and antibiotic additives.

METHODS

Test Drugs

Pharmaceutical tear substitutes were hypromellose (0.3% hydroxypropylmethylcellulose wt/vol) with BAC (0.001% wt/vol; Schering-Plough, Kenilworth, NJ) and unpreserved hypromellose (0.3% wt/vol; Martin...
Epithelial Cell Culture

Primary human corneal epithelial cells were obtained from corneoscleral rims after trephination of corneal grafts at Moorfields Eye bank. After incubation at 37°C for 2 hours with 1.2 IU/ml neutral protease (Dispase II; Boehringer–Mannheim, Mannheim, Germany), the epithelium was stripped off with gentle scraping from the limbus to the center into PBS. This was centrifuged at 100g for 5 minutes, and the cells suspended in K-SFM supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, 5 ng/ml EGF, 2.5 mg/ml bovine pituitary extract (Gibco), and 0.05 mM calcium chloride. The cells were cultured at 37°C with 5% CO2 in 95% humidified air until 80% confluent and were expanded by using routine cell culture techniques. In addition immortalized human corneal epithelial cells (ATCC C11515) from the American Type Culture Collection (ATCC, Rockville, MD) were cultured.

Evaluation of Test Model

Immunohistochemistry with mouse monoclonal antibodies (ICN-flow; ICN, Basingstoke, UK), using the alkaline phosphatase method, stained the cells strongly positive for human cytokeratin-3. The effect of varying volumes (60, 100, and 200 µl) of fluid in the well over 4, 6, and 10 days on adenosine triphosphate (ATP) counts was measured. The pH and osmolality of test drugs were measured before and 2 hours after adjustment to 20°C for unstimulated and 170 IU/ml for stimulated submandibular gland saliva. Amylase activity in a 50-ml sample was determined before and after adjustment using standard freeze-point osmometry (Advanced Microsometree-3; Vitech Scientific, Partridge Green, UK) and a miniature pH meter (pHBoy: Camlab, Cambridge, UK). Human a-amylase type XIII-A (Sigma, Poole, UK) was dissolved in defined keratinocyte serum-free medium (defined K-SFM; Gibco, Paisley, UK) to give activities of 0.8, 4, 20, 50, 100, and 200 IU/ml. In humans, this ranges between 44 IU/ml (Sigma, Poole, UK) was dissolved in defined keratinocyte serum-free medium (defined K-SFM, as well as 2-hour incubation with saliva of varying osmolality or defined K-SFM supplemented with increasing amylase activity, was assessed with the ATP assay only.

Toxicity Studies

All experiments were performed on cells at passages 2 to 4 plated at a density of 15,000 cells/well. The cells were plated either for morphologic evaluation on 16-well glass slides (Nunc, Napierville, IL) or for the ATP assay in 96-well flat bottom plates (Falcon, Plymouth, UK). The cells were incubated for at least 24 hours with K-SFM supplemented with bovine pituitary extract. When approximately 60% confluence was reached, the medium was withdrawn, and the wells were washed twice with PBS. The cells were then incubated for a further 18 hours with defined K-SFM supplemented only with penicillin-streptomycin.

The wells were washed twice with 200 µl PBS and incubated with 200 µl prewarmed single test drug. Each microplate was used to test six to seven single agents as well as a maximum inhibitor (BAC: 1%) and a minimal inhibitor (defined K-SFM in triplicate. The following seven exposure times were used with neat test drug: 10 and 30 minutes, 1, 2, 4, 6, and 24 hours. In analogy to a drop of 38 µl applied to a normal tear volume of 7 µl which is then washed out by the subsequent tear turnover of 1.2 µl/min, the concentration of an initially neat fluid volume after 25 minutes and 1, 2, and 3 hours was calculated. These were 50%, 20%, 4%, and 0.8%. The test drug was diluted in defined K-SFM to these concentrations and applied for 2 hours. These calculations may overestimate the retention time for normal eyes but are likely to be more accurate for absolute aqueous-deficient dry eyes.

Scanning electron microscopy (SEM) was used to evaluate time response. The viability staining and the ATP assay were used to assess time and dose response. The ATP assay was the most sensitive of the three assays used. Therefore, the effect of 10 minutes’ application of test drugs with subsequent PBS wash and 48-hour incubation with defined K-SFM, as well as 2-hour incubation with saliva of varying osmolality or defined K-SFM supplemented with increasing amylase activity, was assessed with the ATP assay only.

Endpoint Assays

For all endpoint assays the test drug was removed and the wells washed twice. For SEM, specimens were fixed in Karnovsky’s fixative, dehydrated through ascending alcohol concentrations, critical point dried, mounted, and sputter coated with 7 to 10 nm gold before examination with a scanning electron microscope (model 6100; JEOL, Tokyo, Japan). The surface morphology of the cells was evaluated at 10 minutes and 6 hours of exposure.

For live-dead viability staining, the cells were incubated with 100 µl of 4 µM ethidium homodimer-1/2 µM calcein-AM (Molecular Probes, Leiden, The Netherlands) for 30 minutes at room temperature. The cells were then immediately viewed with a fluorescence microscope at 485 nm excitation and 515 nm emission wavelength. The nonfluorescent calcein-AM is converted into green fluorescent polyacanionic calcein by intracellular esterase, indicating active cell metabolism. Ethidium homodimer is excluded by viable cells but permeates damaged cell membranes, binds to nucleic acids, and results in red fluorescence. The number of green, red, and bicolored cells was counted in three wells per 10 fields of 315 x 210 µm each at 400-fold magnification. The percentage of cells with exclusive green fluorescence (interpreted as no cell membrane damage) and of green and bicolored fluorescence (interpreted as detectable esterase activity) was calculated.

For the ATP assay all reagents were from DCS Innovative Diagnostik Systeme (Hamburg, Germany). Cellular ATP was extracted by adding 200 µl PBS and 50 µl cell extraction reagent to each well, using a multichannel pipette. The cells were left at least 20 minutes at room temperature before 20 µl of culture extract was transferred and mixed with 50 µl luciferin-luciferase counting reagent, previously equilibrated for 20 minutes to room temperature, into the wells of a white 96-well assay plate (Dynex, Chantilly, VA). The resultant luminescence was read immediately using a luminometer (Dyneatech ML1000; Dynex). An ATP standard curve was performed for all studies using 0.05-ml aliquots of a 250 ng/ml ATP standard serially diluted 1:3 in dilution buffer. All experiments were at least performed twice.

Data Evaluation and Statistical Methods

The percentage of cell growth inhibition (CGI) for each drug and test situation was calculated

\[
1.0 - \frac{\text{Test} - \text{MI}}{\text{MO} - \text{MI}} \times 100 = \%\text{CGI}
\]

where MO is mean counts for no inhibition control cultures, MI is mean counts for maximum inhibition control cultures, and Test is mean counts for triplicate test situation.
The index area under the curve was calculated using the trapezoidal rule. The IC$_{50}$ value is the test drug concentration in culture medium that induces a 50% reduction of ATP levels and was determined by interpolation. Percentage of coefficient of variation was calculated by SD/means. The Wilcoxon signed-rank test for nonparametric data was used to determine the significance of differences. Probabilities were corrected for multiple tests using Bonferroni’s method and were considered to be significant at $P < 0.05$.

**RESULTS**

**Evaluation of Culture and Toxicity Model**

Immunohistochemistry confirmed that the cells were corneal epithelial cells, because they stained strongly for cytokeratin-3. The total ATP count of cell cultures incubated for 4 days in 96-well plates did not depend on the amount of fluid volume covering the cells ($P > 0.95$). The coefficient of variation in the minimal inhibitor wells of the ATP assays was 12%. The osmolality and pH of saliva, serum, and pharmaceutical tear substitutes measured before and after 2 hours’ incubation with the cells remained stable within physiological limits (Table 1).

**Cellular Morphology**

With SEM (Fig. 1) cells cultured for 6 hours with culture medium (minimal inhibitor; Fig. 1B) formed a confluent monolayer and were densely packed with long upright microvilli. Cells that were incubated with 1% BAC (maximum inhibitor; Fig. 1A) for 10 minutes had lost all microvilli and showed large holes in the cell membrane through which the cytoskeleton was visible. Incubation with the test drugs reduced the lawn of microvilli. Hypromellose preserved with BAC (Figs. 1C, 1D) and natural saliva (Figs. 1G, 1H) showed a much more pronounced loss of microvilli than unpreserved hypromellose (Figs. 1E, 1F) or isotonic saliva (Figs. 1I, 1J). Microvilli density of cells cultured in 50% serum (Figs. 1K, 1L) was comparable with negative controls. Because 100% serum was less toxic in the ATP and calcein-AM assay, its effect was not evaluated with SEM. Although these changes were more pronounced for all test drugs after 6 hours’ exposure, this seemed to be more severe with unpreserved hypromellose than isotonic saliva.

**Fluorescent Viability Staining**

Toxicity was defined as an increase in cell membrane permeability and reduction of esterase activity. Figure 2 shows corneal epithelial cells after 2 hours’ incubation with neat test drug; (A) 1% BAC, (B) defined K-SFM, (C, D) 0.3% hypromellose preserved with 0.01% BAC, (E, F) 0.3% hypromellose unpreserved, (G, H) natural saliva, (I, J) isotonic saliva, (K, L) 50% serum for 10 minutes (left column of scans) and 6 hours (right column). Magnification, $\times 1000$; Insets $\times 8000$.

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**Table 1. Osmolality and pH of Saliva, Serum, and Pharmaceutical Tear Substitutes**

<table>
<thead>
<tr>
<th></th>
<th>Before Exposure</th>
<th>After Exposure</th>
<th>Before Exposure</th>
<th>After Exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH Saliva-natural</td>
<td>8.4 ± 0.2 (8.2–8.6)</td>
<td>8.2 ± 0.4 (7.8–8.5)</td>
<td>37 ± 3 (35–39)</td>
<td>47 ± 9 (40–55)</td>
</tr>
<tr>
<td>Saliva-isotonic</td>
<td>8 ± 0.1 (7.9–8)</td>
<td>7.9 ± 0.2 (7.7–8.1)</td>
<td>303 ± 16 (286–317)</td>
<td>311 ± 12 (298–322)</td>
</tr>
<tr>
<td>Serum 50%</td>
<td>7.8 ± 0.1 (7.8–7.9)</td>
<td>7.7 ± 0.1 (7.6–7.8)</td>
<td>279 ± 13 (270–288)</td>
<td>287 ± 13 (277–296)*</td>
</tr>
<tr>
<td>Serum 100%</td>
<td>8.5 ± 0.2 (8.3–8.7)</td>
<td>8.1 ± 0.2 (8.8–1.1)*</td>
<td>291 ± 6 (286–295)</td>
<td>296 ± 6 (292–300)</td>
</tr>
<tr>
<td>Pharmaceutical tear substitutes†</td>
<td>7.9 ± 0.5 (7.5–8.2)</td>
<td>7.9 ± 0.2 (7.7–8.1)</td>
<td>315 ± 4 (312–318)</td>
<td>312 ± 1 (311–318)</td>
</tr>
</tbody>
</table>

Readings were made before and after 2 hours’ exposure on corneal epithelial cells. These parameters remained stable within physiological limits. Ranges appear in parentheses.

* Significant difference between data before and after exposure ($P < 0.05$).
† Mean of hypromellose with and without BAC.
6 hours' incubation ($P < 0.01$) but increased cell membrane permeability after 30 minutes ($P < 0.0001$). Natural saliva altered esterase activity and cell membranes with 30-minute exposures ($P < 0.01$). Adjusting the osmolality of saliva to isotonicity delayed the effect on cell membranes to 2 hours' exposure and esterase extinction to 6 hours ($P < 0.01$). The 50% serum had no effect on esterase ($P > 0.99$) but increased cell membrane permeability after 24 hours ($P < 0.01$). The 100% serum had no significant toxicity ($P = 0.06$).

In a 2-hour incubation of test drug diluted in culture medium, esterase extinction was observed for preserved hypromellose in a concentration of $\geq 20\%$ ($P < 0.00001$). Cell membrane permeability, however, was more sensitive and increased with 4% preserved hypromellose ($P < 0.001$), 50% natural saliva ($P < 0.01$), and 100% unpreserved hypromellose ($P < 0.001$) or isotonic saliva ($P < 0.001$). The 50% ($P < 0.4$) or 100% serum ($P < 0.6$) showed no significant effect at 2 hours of incubation.

ATP Assay

The loss of cellular ATP levels as time and dose responses are shown in Figure 4. At the 2-hour time point, 100% serum resulted in significantly less inhibition than 50% serum or natural saliva. Isotonic saliva was less toxic than natural saliva ($P < 0.01$). Serum in the 50% and 100% preparation ($P < 1 \times 10^{-5}$), as well as isotonic saliva preserved cellular ATP significantly better than unpreserved hypromellose ($P < 0.01$). Natural saliva was not significantly different from unpreserved

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**Figure 2.** Fluorescent viability stain with calcein-AM/ethidium-homodimer. All cells were incubated for 2 hours with 100% of the test drug: (A) Positive control (1% BAC); (B) negative control (culture medium); (C) hypromellose with 0.01% BAC; (D) hypromellose unpreserved; (E) natural saliva; (F) isotonic saliva; (G) 50% serum with 0.5% chloramphenicol; and (H) 100% serum. Magnification, $\times 200$. 

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**Table 1.** Summary of results.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Esterase Activity</th>
<th>Cell Membrane Permeability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural saliva</td>
<td>Decreased</td>
<td>Decreased</td>
</tr>
<tr>
<td>Isotonic saliva</td>
<td>Decreased</td>
<td>Increased</td>
</tr>
<tr>
<td>50% serum</td>
<td>Decreased</td>
<td>Decreased</td>
</tr>
<tr>
<td>100% serum</td>
<td>Decreased</td>
<td>Increased</td>
</tr>
<tr>
<td>Hypromellose</td>
<td>Decreased</td>
<td>Increased</td>
</tr>
<tr>
<td>Hypromellose unpreserved</td>
<td>Decreased</td>
<td>Increased</td>
</tr>
</tbody>
</table>
hypromellose ($P = 0.09$). The 50% serum without chloramphenicol reduced ATP concentrations significantly less than 50% serum with chloramphenicol ($P = 0.049$). Short time exposures of 10 minutes with subsequent incubation in culture medium for 2 days showed no delayed effect of higher dilution of natural tear substitutes. Table 2 gives the figures calculated for the IC$_{50}$ and the area under the dose–response curve (AUC) of all drugs tested in the ATP assay. Table 3 summarizes, categorizes, and ranks the tested tear substitutes.

**Potentially Toxic Properties of Saliva**

Exposure to amylase activities equivalent to physiological stimulated or unstimulated saliva did not change cellular ATP level (Fig. 4G). However, increasing the osmolality of natural saliva up to 300 mOsm (Fig. 4H) reduced its toxicity on primary and immortalized human corneal epithelial cells. At higher osmolalities, there was a trend to increasing toxicity. In 2 hours of incubation, saliva with a physiological osmolality did not reduce ATP levels significantly.

**DISCUSSION**

**Background**

The use of pharmaceutical tear substitutes, especially if preserved with BAC, can be toxic for epithelial and endothelial cells and may result in corneal blindness.$^{8,35–37}$ With increasing severity of the aqueous deficiency, the application frequency of tear substitutes increases, their turnover is reduced, and the ocular surface becomes more susceptible to toxicity.$^{58}$ Therefore, the toxicity of tear substitutes must be minimized for severely aqueous-deficient dry eyes. Unpreserved natural tear substitutes have been advocated in this situation to replace lubrication and nutrition. The current study was conducted to obtain comparative data on the toxicity of natural versus phar-
maceutical tear substitutes as well as to elucidate potential mechanisms of toxicity in natural tear substitutes.

**Validity of Cell Culture Models for Cytotoxicity Tests**

To reduce the number of animals used in the preclinical evaluation of ocular toxicity of substances the Draize test has been substituted by in vitro tests. Cultured epithelial cells of animal and human corneal origins have been used as substrate for these. Monolayered cultures of corneal epithelial cells have been shown to be equally sensitive as three-dimensional corneal constructs for evaluation of acute toxicity. These tests should be performed under standardized conditions, and undefined supplements should be avoided in the culture medium. To allow extrapolation for the clinical situation, human rather than animal and primary rather than immortalized cells should be used.

Limited human corneal donor material for experimental purposes is one reason that in vitro studies are often performed on cultured animal cells. We found corneoscleral rims of short-term, preserved donor material used for keratoplasty a suitable source of primary human corneal epithelial cells. Also, human corneal epithelial cells tend to become senescent after passage which can significantly increase variability and introduce bias. We therefore used a low-calcium medium and performed experiments only on cells up to passage 4. Thus, the coefficient of variation in our model was only 12%, which is

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**FIGURE 4.** Toxicity as loss of cellular ATP in time- and dose-response experiments. The 0% line represents the amount of ATP after incubation with culture medium (minimal inhibitor), and the 100% line is the amount of ATP after incubation with 1% BAC maximum inhibitor (positive control). (A) Time response on incubation with neat test drug: For BAC-preserved hypromellose, \( P < 1 \times 10^{-5} \); for all other test drugs, \( P < 0.01 \). (C) Ten-minute exposure in varying concentrations with subsequent 48 hours of incubation in culture medium: No delayed effect of higher dilution of natural tear substitutes or unpreserved hypromellose on ATP levels was observed. (E, F) Dose response on 2-hour incubation: For BAC-preserved hypromellose, \( P < 0.001 \); for all other tear substitutes, \( P < 0.01 \). ATP response to osmolality or amylase: (G) Cellular ATP measurement after 2 hours' exposure to amylase in defined K-SFM adjusted to varying activities; (H) ATP levels measured in primary and immortalized human corneal epithelial cells after 2 hours' exposure to saliva with the osmolality adjusted to varying levels.
TABLE 2. IC_{50} and Mean Index_{AUC} for All Drugs Averaged Over All Six ATP Assays

<table>
<thead>
<tr>
<th>Test Drug</th>
<th>IC_{50} (%)</th>
<th>Index_{AUC}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum 100%</td>
<td>No inhibition</td>
<td>2.501</td>
</tr>
<tr>
<td>Saliva-isotonic</td>
<td>118</td>
<td>5.545</td>
</tr>
<tr>
<td>Serum 50%</td>
<td>88</td>
<td>6.740</td>
</tr>
<tr>
<td>Hypromellose unpreserved</td>
<td>73</td>
<td>7.241</td>
</tr>
<tr>
<td>Saliva-natural</td>
<td>78</td>
<td>7.767</td>
</tr>
<tr>
<td>Hypromellose with 0.01% BAC</td>
<td>14</td>
<td>15.607</td>
</tr>
</tbody>
</table>

Duplicates of time-response curve, immediate and delayed dose-response curve. Each concentration and time point was evaluated in triplicate. The test drugs are ranked in increasing order of toxicity according to the index_{AUC}. Significant difference: 100% serum versus 50% serum, natural saliva, or unpreserved hypromellose (P < 0.05); isotonic versus natural saliva (P < 0.05); natural saliva versus preserved hypromellose. No significant difference: 100% serum versus isotonic saliva; 50% serum versus natural saliva; unpreserved hypromellose versus natural saliva, isotonic saliva, or 50% serum.

representative of the genetic and phenotypic heterogeneity of normal tissue.34,35

The relevance of in vitro obtained toxicity data is sometimes challenged, because culture conditions cannot substitute for the complex physical and molecular interactions of tear film and ocular surface in vivo. However, in vitro evaluation may allow more appropriate conclusions for severely aqueous-deficient dry eyes, because these are more susceptible to toxicity.36-37 and have a reduced tear turnover. In these evaluations, frequent application of tear substitutes or salivary gland transplantation result in an eye-bath-like exposure. Lastly, test conditions such as drug volume and incubation time were standardized and pH and osmolality controlled and found to be stable within physiological limits. Although cell culture models cannot provide neural pathways that are important in epithelial integrity in the long term, we believe that the model used here is fully defined and allows extrapolation to the acute toxicity of pharmaceutical and natural tear substitutes on human corneal epithelium in vivo.

Relevance of End Point Assays

We assessed and defined toxicity as a loss of cell membrane microvilli and barrier function, as well as intracellular esterase activity and ATP. SEM is an accepted way to visualize drug-induced alterations of the corneal epithelial cell morphology, such as loss of cell coherence and microvilli.41,44,45 However, it is expensive and technically demanding. Fluorescent viability staining provides information beyond morphology, because it visualizes functional alterations of the cell membrane integrity and cell metabolism. It has previously been used to evaluate the viability of corneal donor tissue.32 In our study, cell membrane permeability was a more sensitive parameter than intracellular esterase to determine toxicity in these assays. Tetrazolium salt assays such as the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay have been used extensively to quantify cytotoxicity. However, the luminescence-based ATP assay offers even better sensitivity and reliability for 96-well plate assays.49-51 All three endpoint tests generally agreed in the ranking of the drug toxicity, but the ATP assay was able to quantify a significant cell inhibition after 2 hours of incubation with both serum preparations that was not detected with the viability stain. It also was easier and faster to perform.

Comparison of Cytotoxicity of Pharmaceutical and Natural Tear Substitutes

Our results show that natural tear substitutes are—with the exception of natural saliva—less toxic than pharmaceutical tear substitutes. Autologous serum is used in early clinical studies for aqueous-deficient dry eyes at concentrations of 10% to 100%.24,26 Our data show that undiluted serum maintains ATP levels better than 50% serum. This supports the clinical observation that undiluted serum is required for a sufficient effect in some patients.26 Although reported to be relatively nontoxic, chloramphenicol 0.5% eye drops used to dilute serum to 50% reduced ATP levels more than the addition of culture medium supplemented with penicillin-streptomycin.52 Recent clinical studies suggest that saliva can be beneficial in severely aqueous-deficient dry eyes. Data on the toxicity of saliva on the ocular surface is limited to the report that the incubation of corneal tissue in parotid saliva does not result in histologically detectable enzymatic digestion.53 Our study confirms that amylose, due to its specificity for complex carbohydrates, is not harmful to cultured epithelial cells. The low osmolality of natural saliva was the major factor contributing to its toxicity.

Although moderately hypotonic solutions of 160 mOsm/l reduce the signs and symptoms10,54 caused by the hypertonicity of aqueous-deficient dry eye tears, substitutes of 75 mOsm/l were found to be irritating.55 In rabbits, hypotonic pharmaceutical tear substitutes delayed the recovery of corneal epithelium.10 Our data show that, when incubated for 2 hours with saliva, ATP is best maintained at 180 to 300mOsm/l. Lower or higher osmolalities inhibit cellular metabolism. This can be reproduced in immortalized cells, which confirms the importance of this basic mechanism of toxicity. The toxicity of

TABLE 3. Ranking of Cytotoxicity in Increasing Order after Incubation with 100% Test Drug in All Three Endpoint Assays

<table>
<thead>
<tr>
<th>Test Drug</th>
<th>SEM</th>
<th>Cell Membrane Impermeability</th>
<th>Loss of Esterase Activity</th>
<th>ATP Assay</th>
<th>Sum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum 100%</td>
<td>NT</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Serum 50%/chloramphenicol</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Saliva-isotonic</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Saliva-natural</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>Hypromellose unpreserved</td>
<td>2</td>
<td>4</td>
<td>0</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>Hypromellose + BAC</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>15</td>
<td></td>
</tr>
</tbody>
</table>

For SEM, scores are 0, microvilli as in culture medium; 1, mild; 2, moderate; 3, severe loss of microvilli toxicity; 4, as with 1%. BAC (benzalkonium chloride; maximum inhibitor). For the viability staining and ATP assay, scores are 0, no difference from minimal inhibitor; 1, mild; 2, moderate; 3, severe toxicity; 4, no difference from maximum inhibitor. NT, not tested.
salivary tears provided by gland transplantation is due to surgical denervation and contact with the ocular surface higher (165 ± 74 mOsm; range, 88–310) than in natural saliva.25 It may therefore be less toxic in vivo than unpreserved hypropemellose. However, at the low end of the range, toxicity is likely and may, with continuous lubrication, result in the microcystic epithelial edema observed in selected cases.56

Factors Responsible for the Beneficial Effect of Natural Tear Substitutes

Natural tear substitutes, unlike palliative pharmaceutical tear substitutes, may address parts of the underlying multifactorial pathogenesis of dry eye. Saliva and tears both lubricate mucous membranes. They have similar biochemical and physiological features. For lubrication, both contain mucin and are relatively viscous. For antimicrobial protection, they have similar concentrations of lysozyme, lactoferrin, IgA, and α2-macroglobulin.57–59 To maintain cellular health, they are rich in EGF and vitamin A. These factors can also be found in serum. EGF and fibronectin concentrations in saliva and serum are higher than in normal tears.60,61 However, the concentration of vitamin A in serum is higher (46 μg/ml) than in saliva (51 ng/ml) and this may explain why isotonic saliva is not as effective in maintaining cellular ATP levels as 100% serum.24,63

In summary, our data show that natural saliva has a cytoxic effect on corneal epithelial cells that is comparable to unpreserved pharmaceutical tear substitutes. Iso-osmolar saliva promises better clinical potential for treatment of severe aqueous-deficient dry eyes than do pharmaceutical tear substitutes. Undiluted serum maintains cellular viability best. The addition of antibiotics reduces this effect. Although the use of natural tear substitutes has been anecdotal so far, this approach seems promising for the therapy of aqueous-deficient dry eyes. Determining the beneficial factors responsible for the therapeutic effect of natural tear substitutes on epithelial viability, as well as their required concentration and stability, should be subject of future studies. Human primary corneal epithelial cells cultured under fully defined conditions offer an in vitro model for this, and cellular ATP is a very sensitive parameter to quantify toxicity. Such studies should ultimately allow the formulation of pharmaceutical tear substitutes with equally supportive properties.

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