Nourish and Nurture: Development of a Nutrient Ocular Lubricant

Lei Liu,1 John Tiffany,2 Zexu Dang,1 John K. G. Dart,3 Stephanie L. Watson,3 Julie T. Daniels,4 and Gerd Geerling1,5

PURPOSE. The authors aimed to produce a new tear substitute capable of providing both lubrication and nutrition, based on a novel nutrient-containing therapeutic ocular surface medium (TOSM).

METHODS. Viscous substances, including hypromellose (HPMC), carbopol, and sodium hyaluronate (SH) were added to the TOSM at various concentrations. Three commercial preservative-free artificial tear substitutes, Hypromellose (Pharmacy of Oxford, Oxford, United Kingdom; 3Moorfields Eye Hospital, London, UK), Thilo-Tears (a carbomer; Alcon Pharma GmbH, Freiburg, Germany), and Vislube (a hyaluronate; Chemedica AG, Munich, Germany) were used as control preparations. Their viscosity and surface tension were measured. Human corneal (HCE-T) and conjunctival (IOBA-NHC) cell lines were used to investigate cell proliferation and viability in response to the formulations by means of a luminescence-based ATP assay and a calcein AM/EthD-1 assay.

RESULTS. HPMC, carbopol, and SH increased the viscosity of TOSM significantly. The surface tension of TOSM was reduced by HPMC, but not by carbopol or SH. TOSM-HPMC supported cell proliferation and viability better than TOSM-carbopol and TOSM-SH. TOSM-HPMC and TOSM-carbopol supported cell proliferation significantly better than the corresponding commercial artificial tears. However, TOSM-Vislube supported cell growth significantly better than TOSM-SH.

CONCLUSIONS. TOSM-HPMC showed superior lubricant and nutrient properties with moderate viscosity and little cytotoxicity. It thus could be an ideal nutrient and lubricant tear substitute for dry eyes and should be evaluated in a clinical study.

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and determined their effects on the proliferation and viability of human corneal and conjunctival epithelial cells in vitro.

**Materials and Methods**

**Preparation of Test Solutions**

TOSM was supplemented with HPMC (Dow Chemical Co., Midland, MI), carbolp 980 (Noveon, Inc., Cleveland, OH) and SH (Fluka, Sigma-Aldrich, Chemical Co., Ltd., Poole, UK) to adjust its viscosity. The molecular weight was approximately 1 million Daltons for HPMC and a few million Daltons for carbolp 980. The SH used in this study was extracted from human umbilical cords, and its molecular mass was given as 5 to 5.8 million Daltons by the manufacturer. TOSM was used as a solvent to dissolve all the test substances to concentrations of 0.4%, 0.2%, 0.1%, 0.05%, 0.025%, and 0.0125%. Commercially available unpreserved tear substitutes, Hypromellose (0.3% HPMC; Pharmacy of Moorfields Eye Hospital, London, UK), Thilo-Tears SE (0.3% carbomer; Alcon Pharma GmbH, Freiburg, Germany), and Vislube (0.18% SH, TRB; Chemedica AG, Munich, Germany) were chosen as the control (Table 1).

The pH and osmolarity of all the solutions were measured and adjusted to be close to the physiological value of human tears, with 7.2 to 7.6 for pH and 300 mOsm/L for osmolarity.23 The surface tension and viscosity of TOSM-HPMC, TOSM-carbolp 980, and TOSM-SH at all concentrations were measured immediately after preparation and after 3 months of storage at 4°C to check the stability of their properties. In addition, the three commercial artificial tears were measured for their pH, osmolarity, surface tension, and viscosity.

**Determination of Surface Tension and Viscosity**

The surface tension of the test and control solutions was measured at the Nuffield Laboratory of Ophthalmology (Oxford, UK), with a capillary-microtechnique as was previously described.24 The viscosity of all solutions was measured using a rheometer (Low Shear 30; Contraves, Contraves, Zurich, Switzerland) over the range of shear rates of 1.75 to 128.5 seconds⁻¹, as described by Tiffany.25

**Culture of Human Epithelial Cell Lines**

A human corneal epithelial cell line, HCE-T, and a human conjunctival epithelial cell line, IOBA-NHC, were cultured in 5% CO₂ at 37°C. HCE-T is a human corneal epithelial cell line derived from normal human conjunctival epithelium. The cells were cultured with 1:1 Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen-Gibco, Grand Island, NY) and F12 supplemented with 1 μg/mL insulin, 2 ng/mL EGF, 0.1 μg/mL cholera toxin, 5 μg/mL hydrocortisone, 10% FBS, 50 U/mL penicillin, 50 μg/mL streptomycin, and 2.5 μg/mL amphotericin B. All the medium supplements were purchased from Sigma-Aldrich Co., Ltd. except FBS and the antibiotics, which were from Invitrogen-Gibco.

**Cell Viability/Toxicity: Calcein AM/EthD-1 Assay**

The calcein AM/EthD-1 assay can simultaneously determine live and dead cells with two probes that measure two recognized parameters of cell viability: intracellular esterase activity and plasma membrane integrity. The experiments were performed in triplicate with HCE-T and IOBANHC cells in parallel. TOSM-HPMC, TOSM-carbolp, and TOSM-SH, as well as commercial tear substitutes diluted in TOSM at all the concentrations described herein were tested (Table 1). TOSM and 1% benzalkonium chloride (BAC; Haltermann, Ltd., Workington, UK) were used as positive and negative controls, respectively. Ten thousand cells were seeded per well in black 96-well plates with flat clear bottoms (Costar; Corning, Inc., Corning, NY). After complete confluence, the cells were exposed to the test substances for 24 or 72 hours. After they were washed with PBS twice, the cells were incubated with 150 μL of 4 μM EthD-1/2 μM calcein-AM (Molecular Probes, Leiden, The Netherlands) diluted in PBS for 30 minutes at room temperature. The cells were then viewed and photographed under a fluorescence microscope (Axioskop, Microskop-Kamera MC 100; Carl Zeiss GmbH, Jena, Germany) at 490 nm excitation and 520 nm emission wavelength.

**Cell Proliferation: ATP Assay**

The ATP assay system, described elsewhere, is based on the production of luminescence caused by the reaction of ATP with added luciferase and D-luciferin.15 Dose- and time–response curves were established with a series of doubling dilutions, from 0.4% down to 0.0125%, at 24, 48, 72, 96, and 144 hours of incubation. TOSM as the positive control and 1% BAC as the negative control were used in all experiments which were performed in triplicate and repeated at least once in human corneal and conjunctival epithelial cell line in parallel.

**Data Evaluation and Statistical Methods**

Statistical analysis was performed with commercial software (SPSS for Windows, vers. 11.0.1; SPSS, Chicago, IL). The two-sided t-test was used for osmolarity and surface tension measurements, the Wilcoxon test was used for comparison of viscosity values, the linear-regression test for evaluation of non-Newtonian properties, and the analysis of variance (ANOVA) test for the ATP assay. P ≤ 0.05 was considered statistically significant.

**Results**

**Biophysical Properties of TOSM and Its Modifications**

**pH and Osmolarity.** The pH of TOSM alone, TOSM-HPMC, and TOSM-SH at all the concentrations was 7.2. The pH of commercially available tear substitutes was 7.8 for Hypromellose, 7.1 for Thilo-Tears, and 6.9 for Vislube. TOSM-carbolp solutions were acidic (pH 4.9) and were adjusted to pH 7.2. The osmolarity of TOSM was 278 mOsm/L and that of the TOSM-modifications ranged from 276 mOsm/L (0.025% TOSM-carbolp) to 304 mOsm/L (0.025% TOSM-HPMC). The osmolarity of commercial Hypromellose, Thilo-Tears, and Vislube was 282, 329, and 158 mOsm/L, respectively. As shown in Table 2, the osmolarity of all TOSM-modifications was either slightly lower or within the range of normal tear osmolarities of 299 to 309 mOsm/L.26 A small volume of 2 μL of each solution was measured for osmolarity and the variation of measurements was more likely due to instrument error, for no obvious concentration dependence was observed.
Surface Tension and Viscosity. The surface tension of all solutions was close to that of water (72 mN/m) and TOSM (70.9 mN/m), except for TOSM-HPMC and commercial Hypro-mellose which were obviously lower at around 50 mN/m (Table 3). The viscosity of TOSM, which was ~0.75 mPa·s at all the shear rates, was significantly increased by adding HPMC, carbopol, or SH at all the concentrations tested (P < 0.001; Fig. 1). The observed increase of viscosity was dose dependent with all substances (P < 0.05), although no significant difference was found between 0.2% and 0.4% concentrations of TOSM-HPMC. The viscosity of TOSM with the single viscous compound was comparable to the parallel commercial artificial tear substitutes. The viscosity of commercial Hypromellose, containing 0.3% HPMC, was lower than 0.4% TOSM-HPMC, but higher than 0.2% TOSM-HPMC (P = 0.001). The viscosity of Vislube, containing 0.18% SH, was lower than 0.4% and higher than 0.1% TOSM-SH (P = 0.001), but not different from 0.2% TOSM-SH. Only the viscosity of Thilo-Tears was undetectable, since it was higher than the measuring range of the viscometer. Of all the formulations tested, the viscosity graphs of TOSM-HPMC, TOSM-carbopol, and TOSM-SH at concentrations of 0.4% and 0.2% as well as commercial Hyromellose and Vislube showed non-Newtonian behavior according to a linear regression analysis (P < 0.05). Non-Newtonian rheology is indicated by higher viscosity at lower shear rates and lower viscosity at higher shear rates.

Stability of TOSM Variations. There was no significant difference in pH, osmolarity and ST for 0.4% of TOSM-HPMC, TOSM-carbopol, and TOSM-SH between 24 hours and 3 months after preparation (Table 4). However, Figure 2 shows that the viscosity of 0.4% TOSM-carbopol increased (P = 0.001) and that of 0.4% TOSM-SH decreased (P = 0.001) over 3 months of storage, whereas the viscosity of 0.4% TOSM-HPMC was stable.

Effect of TOSM and Its Modifications on Cell Viability and Proliferation

Cell Viability/Toxicity. There was no noticeable difference of cell viability between the time points of 24 and 72 hours and the images for IOBA-NHC cells exposed to the

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**Table 2. Osmolarity of the Test Solutions**

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Concentrations (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Undiluted 0.0125 0.025 0.05 0.1 0.2 0.4</td>
</tr>
<tr>
<td>TOSM</td>
<td>278</td>
</tr>
<tr>
<td>TOSM-HPMC</td>
<td>298 304 300 291 298 290 —</td>
</tr>
<tr>
<td>Hyromellose</td>
<td>— — — — — — 302</td>
</tr>
<tr>
<td>TOSM-Carbopol</td>
<td>279 276 277 278 284 289 —</td>
</tr>
<tr>
<td>Thilo-Tears</td>
<td>— — — — — — 329</td>
</tr>
<tr>
<td>TOSM-SH</td>
<td>290 294 277 279 282 297 —</td>
</tr>
<tr>
<td>Vislube</td>
<td>— — — — — — 158</td>
</tr>
</tbody>
</table>

Data are in milliosmoles per liter. Manufacturers of commercial preparations are shown in Table 1.

**Table 3. Surface Tension of the Test Solutions**

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Concentrations (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Undiluted 0.0125 0.025 0.05 0.1 0.2 0.4</td>
</tr>
<tr>
<td>TOSM</td>
<td>70.9 — — — — —</td>
</tr>
<tr>
<td>TOSM-HPMC</td>
<td>— 51.1 48.5 45.9 51.8 53.8 53.5</td>
</tr>
<tr>
<td>Hyromellose</td>
<td>51.3 — — — — —</td>
</tr>
<tr>
<td>TOSM-Carbopol</td>
<td>— 71.4 70.5 70.8 73.1 75.3 80.1</td>
</tr>
<tr>
<td>Thilo-Tears</td>
<td>73.1 — — — — —</td>
</tr>
<tr>
<td>TOSM-SH</td>
<td>— 70.7 70.7 70.3 69.8 69.5 68.7</td>
</tr>
<tr>
<td>Vislube</td>
<td>69.2 — — — — —</td>
</tr>
</tbody>
</table>

Data are expressed in millinewtons per meter. Manufacturers of commercial preparations are shown in Table 1.

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**Figure 1.** Viscosity of TOSM-modifications over the range of 1.75 to 128.5 seconds⁻¹ shear rate at 37°C. Higher concentrations of TOSM-modifications tended to be more viscous than did lower concentrations. The viscosity of TOSM with the single viscous compound was comparable to the equivalent commercial artificial tear substitute.

**Figure 2.** Viscosity graphs of TOSM-HPMC, TOSM-carbopol, and TOSM-SH at concentrations of 0.4% and 0.2% as well as commercial Hyromellose and Vislube showed non-Newtonian behavior according to a linear regression analysis (P < 0.05). Non-Newtonian rheology is indicated by higher viscosity at lower shear rates and lower viscosity at higher shear rates.
formulations with concentrations of 0.2% and 0.4% for 72 hours are shown in Figure 3 as examples. The peak toxicity was observed with the highest concentration of each substance. The commercial Hypromellose reduced cell viability after 24 and 72 hours, although no toxicity was observed for TOSM-HPMC. Thilo-Tears showed higher toxicity than 0.4% TOSM-carbopol after 24 and 72 hours. Virtually no decrease in green fluorescence occurred as a result of incubation with 0.2% TOSM-SH or 0.18% Vislube. However, cytotoxicity was found after incubation with 0.4% TOSM-SH after 24 and 72 hours.

**Proliferation.** The mean coefficient of variation for positive control cultures (TOSM) was 11% and was thus within the normal level of biological variation. Figure 4 shows the dose–response of the various ocular surface epithelial cells, as evaluated with the ATP assay after 24 hours of incubation with TOSM-variations. Cell proliferation of both cell types was better supported by TOSM-HPMC than TOSM-carbopol or TOSM-SH ($P < 0.0001$). For TOSM-HPMC at any concentration, the relative cellular ATP level was $\sim 100\%$ or equal to TOSM alone, which indicates that adding HPMC to TOSM, even at 0.4%, did not induce toxicity. However, growth tended to decrease when the cells were incubated with TOSM-carbopol at concentrations of $\geq 0.2\%$ and with TOSM-SH at $\geq 0.1\%$.

The cell growth–supporting ability of TOSM-HPMC, TOSM-carbopol, and TOSM-SH was compared after 24 hours' incubation on IOBA-NHC cells with commercial Hypromellose, Thilo-Tears, and Vislube diluted in TOSM to the equivalent concentrations of the lubricant component. The concentrations from 0.0125% to 0.2% of each group of equivalent formulations were compared. For example, TOSM-HPMC was compared with TOSM-commercial Hypromellose at the same concentration and TOSM-carbopol with TOSM-Thilo-Tears at same concentration. TOSM-HPMC supported cell proliferation significantly better than TOSM-commercial Hypromellose at concentrations of 0.025%, 0.1%, and 0.2% ($P < 0.05$), and TOSM-carbopol better than TOSM-Thilo-Tears at 0.1% and 0.2% ($P < 0.05$), but commercial TOSM-Vislube supported cell growth significantly better than TOSM-SH at concentrations of 0.1% and 0.2% ($P < 0.05$). The comparison with the concentration of 0.2% is shown in Figure 5.

Time-response ATP assays were performed for all supplements in TOSM at concentrations of 0.2% and 0.4% for 24, 48, 72, 96, and 144 hours with HCE-T and IOBA-NHC cells (Fig. 6). TOSM-SH was not included in this assay because in the dose-response assay cells became nonviable when incubated with 0.2% TOSM-SH. Cell growth was reduced in a time-dependent manner with TOSM-carbopol but not with TOSM-HPMC, which still supported approximately 80% cell growth after 144 hours of incubation.
tension less than the critical ST of the ocular surface tends to result in total wetting. The ST of human tears is approximately 43 mN/m and that of the corneal surface is very close to water, which is 72 mN/m.24,27,28 The ST of the TOSM-solution containing HPMC is lower than carbopol and SH, which indicates that in combination with TOSM-HPMC may offer a superior coating ability and minimal disruption of the tear film.29 As expected, HPMC, carbopol, and SH increased the waterlike viscosity of TOSM, even at the lowest concentration tested (0.0125%). The viscosity of 0.4% carbopol was extremely high, with 205 mPa s at 2 seconds−1 and 18 mPa s at 128 seconds−1 shear rate. With such a high viscosity, the solution does not mix with natural tears and is not well tolerated because it makes blinking difficult and forms lumps that disturb vision. A long contact time, achieved by the high viscosity of the tear substitute, is usually favorable, since this reduces the number of applications necessary to control symptoms and signs. Ideally, tear substitutes have the appropriate viscosity to prolong contact time, whereas at the same time, it will not produce dragging sensations and epithelial damage with rapid eye movement. Although the optimal range of viscosity for clinical use has not been stated clearly, mild to moderate viscous tear agents with a viscosity lower than 200 mPa s are best for this purpose.29 TOSM-HPMC with concentrations from 0.2% to 0.4% and TOSM-SH with concentrations of 0.4% demonstrated moderate viscosity and may be suitable to use in a clinical trial, which is necessary to assess the retention time and clinical efficacy of these formulations in vivo.

Besides low surface tension and moderate viscosity, two features are favorable for any lubricant used in a clinical setting. First, it should have a non-Newtonian character and second the formulations should be stable. The viscosity of a non-Newtonian fluid is high under conditions of low shear rate and low under conditions of high shear rate, also described by shear thinning or pseudoplasticity. Normal human tears show shear thinning, with viscosity falling from approximately 5 mPa s at 2 seconds−1 to approximately 1.5 mPa s at 160 seconds−1.25 Non-Newtonian fluids are more comfortable in the eye, since at high-speed blinking non-Newtonian liquid produces less dragging effect on the ocular surface. This finding has led to interest in polymer solutions exhibiting non-Newtonian rheology. Of all the formulations we examined, TOSM-HPMC, TOSM-carbopol, and TOSM-SH at concentrations of 0.2% and 0.4% as well as commercial Hypromellose and Vislube showed non-Newtonian behavior. Our result confirms that 0.2% and 0.4% TOSM-HPMC and 0.4% TOSM-SH may be suitable to use as tear substitutes. The advantage of non-Newtonian solutions is that they offer less drag at the high shear rates experienced in blinking and also offer more resistance to draining at low shear rates (between blinks), and that a Newtonian with the same resistance to draining would have to have a higher viscosity.
throughout the range and hence be more likely to cause drag at blinking speeds. Although our results seem to conflict with findings from Snibson et al.,31 who showed that 0.2% and 0.3% SH had non-Newtonian properties, these cannot be compared directly without specification of either the temperature or the molecular weight of the polymer. Only figures in the shear rate range of 1.75 to 128.5 seconds\(^{-1}\) were recorded in this study and the shear rate associated with normal blinking has been estimated to range from 0 to >40,000 seconds\(^{-1}\).\(^3\) However, limited shear rate range does not matter if viscosity has fallen to its high-shear plateau value within our measuring range. As shear rate rises still higher toward blink speeds, the viscosity will remain

**FIGURE 4.** Dose–response curves of cell growth for HCE-T and IOBA-NHC cells after incubation with TOSM-modifications for 24 hours. Cell proliferation was better supported by TOSM-HPMC than TOSM-carbopol and TOSM-SH \(P < 0.0001\).

**FIGURE 5.** At the concentration of 0.2%, TOSM-HPMC supported cell growth significantly better than TOSM-commercial Hypromellose and TOSM-carbopol better than TOSM-Thilo-Tears after 24 hours’ incubation; however, commercial TOSM-Vislube was significantly more supportive of cell growth than was TOSM-SH.

**FIGURE 6.** Time–response curves of cell growth for HCE-T and IOBA-NHC cells after incubation with TOSM-HPMC and TOSM-carbopol at the concentrations of 0.2% and 0.4% for exposure time from 24 hours to 144 hours. Cell growth was reduced in a time-dependent manner after incubation with TOSM-carbopol and almost complete loss of ATP after 48 hours. TOSM-HPMC had little effect on cell viability after 24 or 48 hours and could still support approximately 80% cell growth after 144 hours’ incubation.
essentially constant, although the shear stress will rise in proportion (viscosity equals stress/strain).

Of the tested solutions, only TOSM-HPMC showed stable biophysical properties, whereas TOSM-carbopol and TOSM-SH were significantly altered after 3 months of storage. Since carbopol tends to form clumps of particles during dissolving, the TOSM-carbopol preparation could still contain some half-hydrated polymers that might have become completely hydrated during the storage, thus further raising the viscosity of the solution. Another hypothesis to explain our findings is that the acrylic acid polymer of carbopol 980 may have cross-linked with components of the base solution (TOSM) to form polymers with even higher molecular weight, which would again increase the viscosity of the solution. The decrease in viscosity of TOSM-SH may be due to some degradation of the SH molecules, since these easily break down into smaller pieces, which then are no longer effective in keeping the viscosity-promoting entanglement. This degradation in viscosity leads to a loss of lubricating ability, resistance to flow, and a reduction of the initial viscosity. Both problems may be overcome in a commercial pharmaceutical production process by appropriate processing or addition of stabilizers.

To determine the least toxic combination, in vitro testing was performed in two different culture models of ocular surface epithelia for all prepared TOSM-modifications. TOSM-SH at 0.1% and higher concentrations induced severe toxicity in the calcine AM/EthD-I assay with nearly 100% cell damage and reduced proliferation in the ATP-assay. However, SH was previously shown to stimulate corneal epithelial cell proliferation and migration. We cannot explain the difference in their observed influence, however, our study showed that the commercial SH cannot be simply prepared in TOSM, since it induced cell toxicity and was also found to be unstable when stored for 3 months. With commercial pharmaceutical production, TOSM-SH is likely to be a formulation worth testing. TOSM-HPMC maintained cell growth better than TOSM-carbopol or TOSM-SH, especially after a longer incubation time. This finding is contradictory to the results of Debbasch et al. who showed that HPMC reduces human conjunctival epithelial cell viability more than SH and carbomer. TOSM-HPMC also supported cell proliferation better than commercial Hypromellose. The cytotoxicity of HPMC may depend on how it is prepared. Purified HPMC, free of protein substances and particulate debris, lowers the possibility of toxicity to living tissue. The product used in our study was highly purified, with a minimum of 94.5% HPMC, which may explain why TOSM-HPMC induced little to no toxicity, even after long-term exposure.

In our study, TOSM supplemented with HPMC at a concentration of 0.2% produced a lubricant with moderate viscosity and good nutrient properties. TOSM-HPMC showed superior physical and nutrient properties compared with other TOSM formulations and any tested commercial product. It was found to have more stable non-Newtonian viscosity over 3 months of storage, suitably low surface tension, and no significant toxic effect in vitro. Unlike other additives, it also does not reduce the epitheliotrophic effect of TOSM. Moreover, HPMC has other potential superior advantages, such as easy availability, ease of preparation, storage at room temperature, and the ability to withstand autoclaving. Therefore, TOSM-HPMC is a potential novel treatment for dry eyes. Further in vivo studies are needed to examine its ocular retention time and efficacy in treating ocular surface disease.

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References


