Effect of Eye Drops Containing Disulfiram and Low-Substituted Methylcellulose Using ICR/f Rats as a Hereditary Cataract Model

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We attempted to develop anti-cataract eye drops using disulfiram (DSF) and low-substituted methylcellulose (MC), and evaluated their anti-cataract effect in terms of the lens opacification vs. age-profile curves using a one-exponential equation. The eye drops were prepared using 0.5% DSF and 2% MC (DSF eye drops), and ICR/f rats, a recessive-type hereditary cataractous strain, were used as the experimental model. Gelation of DSF eye drops containing MC was first observed at about 35°C, close to body temperature. In in vivo transcorneal penetration experiments using rabbit corneas, only diethyldithiocarbamate (DDC) was detected in the aqueous humor, while DSF was not detected. The DDC penetration level of DSF eye drops containing MC was approximately 1.3-fold higher than that of DSF eye drops. The opacification rate constant (k) of ICR/f rat instilled with DSF eye drops with or without MC was lower, and the initial time of opacification (τ) was longer than those of ICR/f rats instilled with saline. Furthermore, the k of ICR/f rats instilled with DSF eye drops with MC was lower than that of ICR/f rats instilled with DSF eye drops without MC. In conclusion, the analysis of kinetic parameters including k and τ using a one-exponential equation provided useful information for clarifying the anti-cataract effect of eye drops. ICR/f rats instilled with DSF eye drops using a low-substituted MC-based drug delivery system demonstrated a delay in cataract development, probably resulting from an increase in the retention of DSF eye drops on the cornea.

Key words cataract; disulfiram; methylcellulose; 2-hydroxypropyl-β-cyclodextrin; ICR/f rat

A cataract is defined as any alteration in the optical homogeneity of the lens or decrease in its transparency, and numerous factors have been implicated in the etiology of cataracts including genetic factors, diabetes, smoking, nutrition, the cumulative effect of X-rays, UV irradiation, and alterations in both endocrine and enzymatic equilibria. In human senile cataracts, an ionic imbalance in the lens with increased levels of Ca2+ is related to cataract development. An elevated Ca2+ content in the lens has been shown to activate calpain, a Ca2+-dependent protease. Furthermore, the degradation of lens proteins such as crystallin proteins results in an opaque lens. Over the past several decades, there have been many studies exploring the mechanisms of cataract development; however, no potent anti-cataract drugs have yet to be introduced due to uncertainties regarding efficacy and safety.

Disulfiram (DSF), a dimer of diethyldithiocarbamate (DDC), has long been used to treat alcoholic syndrome without severe side effects. DSF is a powerful antioxidant that scavenges reactive oxygen species including hydroxyl radicals, superoxide, and peroxynitrite, and chelates metal ions in rats. However, its application in the ophthalmic field is limited due to its poor water solubility. One problem with lipophilic drugs, such as DSF, administered as aqueous eye drops is obtaining the desired drug concentration in any drug delivery system. Cyclodextrins are cyclic oligosaccharides comprising R-δ-glucose linked by R(1→4) glucosidic bonds. The potential use of natural cyclodextrins and their synthetic derivatives has been studied extensively to improve certain properties such as their solubility, stability, and/or bioavailability. 2-Hydroxypropyl-β-cyclodextrin (HPβCD) is a cyclic oligosaccharide with a hydrophilic outer surface and a lipophilic cavity at its centre, and is capable of forming inclusion complexes with many lipophilic drugs by taking up the drug molecule, or part of it, into the lipophilic cavity. In aqueous solution, hydroxypropylmethylcellulose (HPMC), a watersoluble polymer, increases the solubilizing effect of cyclodextrins on lipophilic drugs by increasing the stability constants of the drug/cyclodextrin inclusions. In a previous study, we prepared a HPβCD solution containing DSF and HPMC (DSF eye drops), and found that the instillation of these DSF eye drops delayed lens opacification in ICR/f rats, a recessive-type hereditary cataractous strain. Therefore, it is possible that the antioxidant effect of DSF eye drops may provide a means to prevent or delay the formation of cataracts, and so it is very important to increase the efficacy of DSF eye drops. In this study, we attempted to enhance the delay effect of cataract development of DSF eye drops using low-substituted methylcellulose (MC, METLOSE SM-4). The anti-cataract effect of the new eye drops was evaluated in terms of the kinetic parameters of the lens opacification vs. age-profile curves using a one-exponential equation.

MATERIALS AND METHODS

Animals Male ICR/f rats (mutant) and adult Japanese albino rabbits, 2.5—3.0 kg, were used in this study. They were housed under standard conditions (12 h light/12 h dark, fluorescent light (07:00—19:00), 25°C room temperature) and allowed free access to a commercial diet (CE-2 or CR-3, Clea Japan Inc., Tokyo) and water. All procedures were performed in accordance with the Kinki University School of Pharmacy Committee Guidelines for the Care and Use of Laboratory Animals and the Association for Research in Vision and Ophthalmology resolution on the use of animals in research.

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Table 1. Formulations of DSF Eye Drops with or without MC

<table>
<thead>
<tr>
<th>Content (%)</th>
<th>DSF</th>
<th>HPβCD</th>
<th>HPMC</th>
<th>MC</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSF eye drops</td>
<td>0.5</td>
<td>5.0</td>
<td>0.1</td>
<td>—</td>
</tr>
<tr>
<td>DSF eye drops with MC</td>
<td>0.5</td>
<td>5.0</td>
<td>0.1</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Reagents DSF was kindly donated by Ouchi Shinko Chemical Industrial Co., Ltd. (Tokyo, Japan). HPβCD (average molar substitution, 0.6; average MW, 1380) was purchased from Nihon Shokuhin Kako Co., Ltd. (Tokyo, Japan). HPMC and low-substituted methylcellulose (MC, METLOSE SM-4, average viscosity, 4 Pa·s in 20°C) were provided by Shin-Etsu Chemical Co., Ltd. (Tokyo, Japan). Benzalkonium chloride was obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). All other chemicals used were of the highest purity commercially available.

Preparation of Eye Drops Containing DSF and MC HPβCD was added to saline containing 0.005% benzalkonium chloride with DSF, and then HPMC was added to the solution. The mixture was stirred for 24 h in the dark at room temperature, and filtered through a Minisart CE (pore size of 0.20 μm, Costar Co., Massachusetts, U.S.A.). DSF adsorption was not observed during filtration. Sterilized saline containing MC was added to the DSF solution. The compositions of the DSF eye drops with or without MC are shown in Table 1. The 0.5% DSF is equivalent to 16.9 mm DSF.

Measurement of Gelation of DSF Eye Drops with or without MC DSF eye drops with or without MC as described in Table 1 were heated from 20—55°C, and transmittance was measured by using a UV-2200 (SHIMADZU) at 560 nm. The temperature of gelation of MC was expressed as transmittance (%).

In Vitro Transcorneal Penetration of DDC from DSF Eye Drops with or without MC The in vitro transcorneal penetration of DDC from DSF eye drops with or without MC was examined using the method of Iwata et al. 22) Euthanasia of adult Japanese albino rabbits, weighing 2.5 to 3.0 kg, were done by injection of a lethal dose of pentobarbital into the marginal ear vein. The eyes were removed and the corneas were carefully separated from other ocular tissues. The individual corneas were placed on a methacrylate cell designed for transcorneal penetration studies. The side of the chamber (donor chamber) exposed to the exterior surface of the cornea was filled with DSF eye drops with or without MC. The other side of the chamber (reservoir chamber) was filled with 10 mm4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) buffer (pH 7.4) containing 136.2 mm NaCl, 5.3 mm KCl, 1.0 mm K2HPO4, 1.7 mm CaCl2, and 5.5 mm glucose. The experiments were performed at 35°C for 6 h. Fifty microliters of sample solution was withdrawn from the reservoir chamber at the indicated time intervals and replaced with the same volume of buffer. DSF and DDC concentrations in the samples were determined by the HPLC method. Fifty microliters of sample solution was added to 100 μL methanol containing 0.25 μg indomethacin (internal standard), and the mixture solutions were filtered through a Chromatodisk 4A (pore size of 0.45 μm, Kurabo Industries Ltd., Osaka, Japan). An aliquot of the filtrated solution (10 μL) was injected into a Mightysil RP-18 (3 μm, column size: 2.0 mm × 50 mm) column (Kanto Chemical Co., Inc., Tokyo, Japan) using a Shimadzu LC-10AD system equipped with a column oven CTO-6A (Shimadzu Corp., Kyoto, Japan). The mobile phase consisted of 45% acetonitrile containing 0.1% trifluoroacetic acid. The flow rate was 0.25 mL·min−1, the column temperature was 35°C, and the wavelength for detection was 215 nm. 22) The viability of the corneas was monitored by measurements of thickness or weight (no significant changes in thickness or weight were observed during the 6 h period).

The obtained data were analyzed using following equation (Eqs. 1—3) 22):

\[ \tau = \frac{\delta^2}{6D} \]  

\[ J_c = \frac{K_m \cdot D \cdot C_{DSF}}{\delta} = K_p \cdot C_{DSF} \]  

\[ Q_t = J_c \cdot A \cdot (t - \tau) \]

where \( J_c \) is the DDC penetration rate, \( k_m \) is cornea/preparation partition coefficient, \( D \) is the diffusion constant within the cornea, \( C_{DSF} \) is the DSF concentration in DSF eye drops, \( \tau \) is the lag time, \( \delta \) is thickness of the cornea (0.0625 cm, average for 4 rabbits), \( Q_t \) is the total amount of DDC appearing in the reservoir solution at time \( t \), and \( A \) is the effective area of cornea (0.78 cm²); \( J_c \) and \( \tau \) were estimated by fitting each penetration profile to Eq. 3. The penetration coefficient through the cornea, \( k_p \), is given by \( J_c / C_{DSF} \). A nonlinear least-squares computer program (MULTI) was applied for the calculation. 22)

In Vivo Transcorneal Penetration of DDC from DSF Eye Drops with or without MC The in vivo transcorneal penetration of DSF from DSF eye drops was determined as described by Meisner et al. 23) and Ito et al. 24) Adult Japanese albino rabbits weighing 2.5 to 3.0 kg were anesthetized by injecting pentobarbital (0.6 mg·kg⁻¹) through the marginal ear vein, and a topical anesthetic (0.4% Benoxil) was instilled into each eye 3 min before sampling of the aqueous humor. Then, a 29 gauge injection needle connected to silicon tubing (inner diameter: 0.5 mm, Fuji Systems Co., Tokyo, Japan) joined to a 25 μL microsyringe (Ito Corp., Tokyo, Japan) was inserted into the eye to obtain aqueous humor samples, and remain to stabilize for 30 min. The 50 μL of DSF eye drops was instilled into the eyes of the rabbits. Aqueous humor samples (5 μL each) were removed periodically from the anterior chamber of the eye over the course of 0—90 min from the installation of the eye drops. DSF and DDC concentrations were determined by HPLC as described above.

The DDC concentration data in the aqueous humor after a single injection of 20 μL of DDC solution into the anterior chamber of the eye were analyzed according to Eq. 4 25):

\[ C_{AH} = C_0 \cdot e^{-k_p \cdot t} \]  

where \( C_{AH} \) is the DDC concentration in the aqueous humor at time \( t \), \( C_0 \) is the initial concentration of DDC in the aqueous humor, and \( k_p \) is the DDC penetration rate through the cornea.
were randomly divided into three groups. Two groups, respectively, while the third group received saline. One drop of DSF eye drops with or without MC, were administered DSF eye drops with or without MC, re-

were kept open for about 1 min to prevent the DSF eye drops from overflowing. Image analyses of cataract development were performed as described by Ito et al. The pupils of the ICR/f rats were dilated by the instillation of 0.1% pivalephrine (Santen Pharmaceutical Co., Osaka, Japan) without anesthesia. Changes in the transparency of the lenses were monitored using an EAS-1000 equipped with a CCD camera (Nidek, Aichi, Japan). The outline of the lens image was determined by selecting 4 points on the image, and then the transparent area within the outline and thread level were set automatically by the software. The total area of opacity of the lenses, expressed as pixels, was calculated using the following equation (Eq. 7):

\[
\text{Pixels within opacity (Pixel)} = \text{pixels within outline} - \text{pixels within transparent area}
\] (7)

The effect of DSF eye drops on lens opacification is represented by the opacification rate constant \(k_\text{d} \text{ d}^{-1}\). The opacification rate constant, \(k_\text{d}\), was analyzed according to following equation (Eq. 8):

\[
O_t = O_\infty \cdot (1 - e^{-k_\text{d} \cdot t})
\] (8)

in which \(O_\infty\) and \(O_t\) are the areas of opacity of the lenses at times \(\infty\) and \(t\). The \(O_\infty\) value (mature opacity) obtained in 10 experiments was 16103 pixels. \(t\) (days) shows the initial time of opacification, and \(t_{50\%}\) (days) is time at 50% of \(O_\infty\).

**Statistical Analyses**

All values are represented as mean±standard error of the mean (S.E.). Unpaired Student’s \(t\)-tests were used to evaluate statistical differences, and multiple groups were evaluated by one-way analysis of variance followed by Dunnett’s multiple comparison. \(p\) values less than 0.05 were considered significant.

**RESULTS**

**Gel Formation of DSF Eye Drops with or without MC**

Figure 1 shows the relationship between transmissivity and temperature for DSF eye drops with or without MC at 560 nm. The transmissivity of DSF eye drops containing saline did not change, while the transmissivity of the eye drops containing MC decreased at temperatures greater than 35°C. The transmissivity of DSF eye drops either with and without MC was clear at room temperature without any viscosity; however, the transmissivity of DSF eye drops with MC decreased from about 35°C, close to body temperature, and gelation was observed. No decrease in the transmissivity in DSF eye drops without MC was observed.

**Transcorneal Penetration of DDC from DSF Eye Drops with or without MC**

Figure 2 shows in vitro transcorneal penetration of DDC from DSF eye drops with or without MC using rabbit corneas, and Table 2 summarizes the pharmacokinetic parameters calculated from the in vitro transcorneal penetration data. In the reservoir chamber, only DDC was detected (DSF was not detected), and the amount of DDC in the reservoir chamber increased with time for DSF eye drops both with and without MC. On the other hand, both the \(k_{1/2}\) and \(\tau\) of DSF eye drop with MC were higher than those of DSF eye drops. In addition, \(D\) for the DSF eyedrops with MC was also lower than that of the eye drops without MC. However, no significant differences in the \(J_{\text{f}}\) values (DDC penetration rate) were found for DSF eye drops either with or without MC. Figure 3 shows the DDC concentrations in the aqueous humor after the instillation into rabbit eyes of DSF eye drops with or without MC, and Table 3 summarizes the pharmacokinetic parameters calculated from the in vivo transcorneal penetration data. In the aqueous humor, only DDC was detected, with a peak concentration observed 20 min after eye drop instillation. In contrast to the in vitro transcorneal penetration results, the DDC concentration in the aqueous humor after the instillation of DSF eye drops with MC was significantly higher than that of DSF eye drops without MC, and the \(AUC_{\text{DDC}}\) values for DSF eye drops with MC were approximately 1.3-fold higher than for DSF eye drops without MC.

**Effects of DSF Eye Drops with or without MC on**
Cataract Development in ICR/f Rat

Figure 4 depicts Scheimpflug slit images documented by EAS-1000 (A), and the delaying effect (B) of the instillation of DSF eye drops with or without MC on cataract development in ICR/f rats. From 35 to 63 d of age, the lenses of ICR/f rats remained transparent. Lens opacification in control ICR/f rats instilled with saline began at 77 d of age, and mature cataracts had formed at 84 d. On the other hand, the lenses of ICR/f rats instilled with DSF eye drops either with or without MC were less opaque than those of ICR/f rats instilled with saline. Table 4 summarizes the pharmacodynamic parameters calculated from the lens opacification vs. age-profile curves using a one-exponential equation. The k values for ICR/f rats instilled with DSF eye drops with or without MC were lower, and the τ and t50% values were higher than those of ICR/f rats instilled with saline. Furthermore, the k value for ICR/f rats instilled with DSF eye drops with MC was significantly lower than that of ICR/f rats instilled with DSF eye drops without MC.

DISCUSSION

MC is a water-soluble substance with a high degree of purity, uniformity, and transparency. MC solutions are neutral, odorless, and tasteless. MC solutions are also stable over the pH range that is well tolerated by the eye, and its effectiveness in solutions to treat dry eye, punctate keratitis, and epithelial defects has been reported.25) In this study, we attempted to enhance the delaying effect of DSF eye drops on cataract development by using low-substituted MC. The anti-catarract effect of the drops was evaluated by a kinetic parameter analyzed from the lens opacification vs. age-profile curves using a one-exponential equation.

For eye drops, safety is an important factor. It has been reported that HP/CD ranks second in safety to γ-CD in a variety of CD derivatives used for eye drop applications.26) Jansen et al.27) reported no irritation to eye membranes by HP/CD solutions less than 12.5%. Therefore, we used 5% HP/CD, which is low in comparison with the concentrations used in that report, and determined the DSF concentrations to be used. Finally, 2% MC, a clinically concentration, was added to the DSF eye drops.

In both in vitro and in vivo transcorneal penetration experiments using rabbit corneas, only DDC was detected in the resin.

Table 2. Pharmacokinetic Parameters for the in Vitro Transcorneal Penetration of DDC Released from DSF Eye Drops with or without MC

<table>
<thead>
<tr>
<th></th>
<th>Jc (nmol·cm⁻²·min⁻¹)</th>
<th>kp (×10⁻⁴·min⁻¹)</th>
<th>km (×10⁻³)</th>
<th>τ (min)</th>
<th>D (×10⁻³·cm⁻²·min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSF eye drops</td>
<td>1.64±0.04</td>
<td>5.78±0.03</td>
<td>5.4±0.1</td>
<td>0.97±0.49</td>
<td>67.4±34.2</td>
</tr>
<tr>
<td>DSF eye drops with MC</td>
<td>1.49±0.05</td>
<td>5.71±0.11</td>
<td>22.5±0.3*</td>
<td>4.11±2.34</td>
<td>15.8±7.2</td>
</tr>
</tbody>
</table>

DSF eye drops, 0.5% DSF eye drops; DSF eye drops with MC, 0.5% DSF eye drops with 2% MC. The data are presented as the means±S.E. of 4 rabbit corneas. *p<0.05, vs. DSF eye drops.

Table 3. Pharmacokinetic Parameters for the in Vivo Transcorneal Penetration of DDC Released from DSF Eye Drops with or without MC

<table>
<thead>
<tr>
<th></th>
<th>AUC_DDC (μg·min)</th>
<th>kₚ (min⁻¹)</th>
<th>τ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSF eye drops</td>
<td>3275±148</td>
<td>0.083±0.008</td>
<td>4.96±0.18</td>
</tr>
<tr>
<td>DSF eye drops with MC</td>
<td>4290±214*</td>
<td>0.104±0.007*</td>
<td>4.65±0.22 *</td>
</tr>
</tbody>
</table>

DSF eye drops, 0.5% DSF eye drops; DSF eye drops with MC, 0.5% DSF eye drops with 2% MC. AUC_DDC, the area under the DDC concentration-time curve; kₚ, absorption rate constant; τ, lag time. The data are presented as means±S.E. of 5 independent rabbits. *p<0.05, vs. DSF eye drops.
A reservoir side or aqueous humor. We previously reported that this in vivo transcorneal penetration experiment using rabbit don't affect to a factor in aqueous humor such as nitric oxide (NO), and can apply to the pharmacokinetic evaluation of the eye drops.18,29) In addition, we found that a sulfhydryl-rich protein, aldehyde dehydrogenase 3A1 (ALDH3A1), that is related to

Fig. 4. Effect of DSF Eye Drops with MC on Cataract Development in ICR/f Rats.

Scheimpflug slit images (A) and lens opacity (B) of lenses of ICR/f rats treated by instillation of DSF eye drops with or without MC. Control, saline-instilled ICR/f rat (○); DSF, 0.5% DSF eye drops-instilled ICR/f rat (▲); DSF-MC, 0.5% DSF eye drops with 2% MC-instilled ICR/f rat (■). The numbers above the photographs show the ages of the rats (days). The data are presented as the means±S.E. of 8 independent rats. *1p<0.05, vs. Control. *2p<0.05, vs. DSF eye drops.

Table 4. Pharmacodynamic Parameters for Lens Opacification in ICR/f Rats Instilled with 0.5% DSF Eye Drops with or without MC

<table>
<thead>
<tr>
<th>Treatment</th>
<th>k (×10⁻²·d⁻¹)</th>
<th>t_{50%} (d)</th>
<th>τ (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.94±0.48</td>
<td>77.6±1.4</td>
<td>70.4±0.4</td>
</tr>
<tr>
<td>DSF eye drops</td>
<td>4.27±0.30*</td>
<td>83.4±1.7*</td>
<td>71.6±0.4*</td>
</tr>
<tr>
<td>DSF eye drops with MC</td>
<td>3.41±0.21***</td>
<td>90.8±2.1***</td>
<td>72.3±0.7*</td>
</tr>
</tbody>
</table>

Control, saline-instilled ICR/f rat; DSF eye drops, 0.5% DSF eye drops-instilled ICR/f rat; DSF eye drops with MC, 0.5% DSF eye drops with 2% MC-instilled ICR/f rat. k, opacification rate constant; t_{50%}, time at 50% of matured opacity; τ, initial time for opacification. The data are presented as the means±S.E. of 8 independent rats. *p<0.05, vs. Control. **p<0.05, vs. DSF eye drops.

B

ervor side or aqueous humor. We previously reported that this in vivo transcorneal penetration experiment using rabbit don’t affect to a factor in aqueous humor such as nitric oxide (NO), and can apply to the pharmacokinetic evaluation of the eye drops.15,29) In addition, we found that a sulfhydryl-rich protein, aldehyde dehydrogenase 3A1 (ALDH3A1), that is related to
the conversion of DSF to DDC, and exists in abundance in the corneal stroma and endothelium.\textsuperscript{8,28,29} This suggests that DSF in HP/CD solutions containing HPMC or MC is converted to DDC by ALDH3A1 in the cornea. No significant difference was found in the amount of DDC penetration between DSF eye drops with and without MC in the \textit{in vitro} transcorneal penetration experiments (Fig. 2). In contrast, in the \textit{in vivo} transcorneal penetration experiments, the DDC concentration in the aqueous humor after the instillation of DSF eye drops with MC was significantly higher than when DSF eye drops without MC were instilled (Fig. 3), and the $AUC_{DDC}$ value of the DSF eye drops with MC was approximately 1.3-fold higher than that of the DSF eye drops without MC (Table 3). In the \textit{in vitro} transcorneal penetration experiments, the viscosity and corneal contact area of the DSF eye drops either with or without MC were almost the same, since the eye drops were completely stirred in the chamber. These results suggest that transcorneal DDC penetration is regulated by the viscosity and corneal contact area of the DSF eye drops, and that the addition of MC into the eye drops may result in the increase in the viscosity and corneal contact area. It is known that the eye drops are lost from the tear film within 30—2 min, while a small amount remains associated with the conjunctival tissue.\textsuperscript{30} On the other hand, MC coagulates upon heating, and redissolves upon cooling, and we demonstrated the gelation of DSF eye drops containing MC from about 35°C, near body temperature (Fig. 1). In addition, Schultz et al. reported that when MC-based eye drops are applied, approximately 90% of the drops are lost from the tear film within 10 min, while a small amount (approximately 10%) remains associated with the conjunctival tissue.\textsuperscript{30} These findings suggest that the instillation of DSF eye drops with MC leads to gelation, and that the gelation increases the retention time of the eye drops on the surface of the cornea. The increased retention time of DSF eye drops with MC may result in the higher $AUC_{DDC}$ in comparison with DSF eye drops without MC.

In studies to develop anti-cataract drugs, the selection of the experimental animal is very important. The ICR/f rat is a recessive-type hereditary cataractous strain. Opacity in the experimental animal is very important. The ICR/f rat is an appropriate animal to study the delay of cataract development.\textsuperscript{19} Therefore, we investigated the effect of DSF eye drops on cataract development in ICR/f rats, probably by increasing the retention time of the DSF eye drops on the cornea. The Ca\textsuperscript{2+} content in the cataractous lenses of ICR/f rats is about 10-fold higher than in the lenses of Wistar rats, and autolytic products formed by calpain can also be detected in cataractous lenses.\textsuperscript{33,34} We also reported that excessive nitric oxide produces an increase in lipid peroxide, and that the elevated lipid peroxide levels cause an increase in Ca\textsuperscript{2+}-ATPase activity and increase in Ca\textsuperscript{2+} content in ICR/f rat lenses during cataract development.\textsuperscript{18,19} It is noteworthy that ICR/f rat cataracts are not related to diabetic cataracts, and that the mechanism of cataract development is similar to that of senile cataracts. Furthermore, our previous study using ICR/f rats showed that the DSF eye drops have the ability to attenuate the increase in the NO and LPO levels, resulting in a delay in cataract development.\textsuperscript{19} Therefore, we investigated the effect of DSF eye drops containing MC on cataract development in ICR/f rats in terms of kinetic parameters ($k$, $\tau$, and $t_{50\%}$) analyzed from the lens opacification vs. age-profile curves using a one-exponential equation. The $k$ value was decreased by the instillation of DSF eye drops, while the values of $\tau$ and $t_{50\%}$ of ICR/f rats instilled with DSF eye drops were higher than those of ICR/f rats instilled with saline. Furthermore, these changes in $k$, $\tau$ and $t_{50\%}$ were enhanced by the addition of MC (Table 4, Fig. 4). These results suggest that the instillation of DSF eye drops delays the progression of cataract development in ICR/f rats, and that the delaying effect is increased by the addition of MC into the DSF eye drops.

It is important to clarify the site of action in the instillation of DSF eye drops. We previously reported that liposomes containing DSF after its instillation into rat eyes are absorbed though the nasal mucosa into the blood, and then DDC is detected in the plasma. The elevated plasma DDC concentration prevents cataract development in selenite-treated rats.\textsuperscript{12} In fact, the instillation of liposomes containing DSF into one eye only suppresses the opacification of both eyes in the case of selenite-treated rats.\textsuperscript{12} However, in the present study, the instillation of DSF eye drops into right eye had no effect on the opacification of the left eye in ICR/f rats (data not shown). Therefore, the delaying effect of DSF eye drops may affect only the treated eye. On the other hand, the $C_{max}$ was approximately 20 min after instillation of DSF eye drops in \textit{in vivo} transcorneal penetration experiments using rabbit, and the almost eye drops are lost from the aqueous humor within 2 h. However, the instillation of DSF eye drops at twice a day showed the delay effect of opacification in the ICR/f rats. Taken together, we hypothesize that the inhibited NO and LPO by DDC don’t reduce immediately after the loss of DDC in the lens.

Further studies are needed to elucidate the precise mechanisms of the anti-cataract effect of DSF eye drops. Therefore, we are now investigating the relationship between DDC concentration and inhibition of NO and LPO in human lens cells using a human lens epithelial cell line, SRA01/04.\textsuperscript{35}

In conclusion, DSF eye drops in a low-substituted MC-based drug delivery system promote the delay of cataract development in ICR/f rats, probably by increasing the retention time of the DSF eye drops on the cornea. We conclude that the topical application of DSF containing low-substituted MC is a stable formulation to enhance the anti-cataract effect of DSF eye drops. In addition, the analysis of kinetic parameters such as $k$, $\tau$ and $t_{50\%}$ using a one-exponential equation provides useful information for clarifying the anti-cataract effect of eye drops.

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