Reduction in Intraocular Pressure by the Instillation of Eye Drops Containing Disulfiram Included with 2-Hydroxypropyl-β-cyclodextrin in Rabbit

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We have studied the effect of disulfiram (DSF) solution containing 2-hydroxypropyl-β-cyclodextrin and hydroxypropylmethylcellulose (DSF eye drops) on intraocular pressure (IOP) in experimentally induced ocular hypertension in rabbits. In both in vitro and in vivo transcorneal penetration experiments using rabbit corneas, only diethylthiocarbamate (DDC) was detected in the aqueous humor, while DSF was not detected. The amount of DDC penetration for 0.25% DSF eye drops was about 4-fold that for 0.1% DSF eye drops in in vivo transcorneal penetration experiments. The elevation in IOP was induced by the rapid infusion of 5% glucose solution (15 ml/kg of body weight) through the marginal ear vein, and IOP was measured with an electronic tonometer. The induced elevation in IOP was reduced by the instillation of 0.1—0.5% DSF eye drops, and the IOP-reducing effect increased with the increase in DSF concentration in the drops. Nitric oxide (NO) levels increased in the aqueous humor following the infusion of the 5% glucose solution, and this increase was also suppressed by the instillation of DSF eye drops. In conclusion, the present study demonstrates that the instillation of DSF eye drops has an IOP-reducing effect in rabbits with experimentally induced ocular hypertension, probably caused by the suppression of NO production.

Key words: glaucoma; nitric oxide; disulfiram; 2-hydroxypropyl-β-cyclodextrin; hydroxypropylmethylcellulose

Glaucoma is characterized by nerve degeneration that causes the disappearance of retinal ganglion cells, visual field loss and excavation of the optic disk, and ophthalmopathy.1,2) It is one of the most common causes of visual impairment and blindness throughout the world and is more common in the elderly.3) The major risk factor for glaucoma is elevated intraocular pressure (IOP), which leads to apoptosis and loss of retinal ganglion cells.4) In treating glaucoma, the focus is on reducing IOP, and retinal and optic nerve damage. However, the retinal and optic nerve damage that result from elevated IOP are not satisfactorily controlled by the current therapies. Therefore, the search for successful therapies for glaucoma is a high priority.

Nitric oxide (NO) is synthesized from the guanidino-nitrogen of L-arginine and molecular oxygen by nitric oxide synthase (NOS). Endothelial NOS and neuronal NOS are present in most ocular tissues, including those responsible for aqueous dynamics, i.e. the ciliary processes, ciliary muscle and trabecular meshwork.5—7) NO causes relaxation of the ciliary muscle and trabecular meshwork.8,9) The relaxation of the ciliary muscle tends to decrease trabecular outflow facility and increase uveoscleral outflow, while relaxation of the trabecular meshwork increases trabecular outflow facility. Thus the effects of NO on IOP vary depending upon the site of action. On the other hand, recently, Kiel et al.10) reported that the systemic inhibition of NOS by NG-nitro-L-arginine methyl ester (L-NAME) causes a large, rapid decrease in IOP by due to ciliary vasoconstriction in rabbits. Therefore, agents that inhibit NOS in the blood vessel of ciliary body might prove useful in the treatment of glaucoma.

Diethylthiocarbamate (DDC) is a potent NOS inhibitor and radical scavenger.11,12) However, DDC is unstable in neutral solution, and is not able to penetrate through the cornea into the aqueous humor.13) Disulfiram (DSF), a dimer of DDC, has long been used to treat alcoholic syndrome without severe side effects.14) However, its application in the ophthalmic field is limited due to its poor water solubility.

Cyclodextrins are cyclic oligosaccharides comprising R-α-glucose linked by R-(1—4) glucosidic bonds. Natural cyclodextrins and their synthetic derivatives have been studied extensively to improve certain properties such as their solubility, stability, and/or bioavailability.15) 2-Hydroxypropyl-β-cyclodextrin (HPβCD) is a cyclic oligosaccharide with a hydrophilic outer surface and a lipophilic cavity that is capable of forming inclusion complexes with many lipophilic drugs by taking up the drug molecule, or part of it, into its lipophilic cavity.16,17) In aqueous solution, hydroxypropylmethylcellulose (HPMC), a water-soluble polymer, increases the solubilizing effect of cyclodextrins on lipophilic drugs by increasing the stability constants of the drug/cyclodextrin inclusions.18) We previously found that HPMC increased the solubility of DSF, and solve the problem of its poor water solubility.19) In this study, we investigated the effect of DSF solutions containing HPβCD and HPMC (DSF eye drops) on IOP in rabbits.

MATERIALS AND METHODS

Animals Male Japanese albino rabbits, 2.5—3.0 kg, were used in this study. They were housed under standard conditions (12 h/d fluorescent light (07:00—19:00), 25°C room temperature) and allowed free access to a commercial diet (CR-3, Clea Japan Inc., Tokyo) and water. All procedures were performed in accordance with the Kinki University Faculty 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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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 was provided by Shin-Etsu Chemical Co., Ltd. (Tokyo, Japan). Benzalkonium chloride was obtained from Kanto Chemical Co., Inc. (Tokyo, Japan), and 0.4% Benoxil was provided by Santen Pharmaceutical Co., Ltd. (Osaka, Japan). All other chemicals used were of the highest purity commercially available.

Preparation of Eye Drops Containing DSF Included with HPβCD  HPβCD was added to saline containing 0.005% benzalkonium chloride along with DSF fine powder, and then the 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, Cambridge, MA, U.S.A.). The adsorption of DSF was not observed by filtration. The compositions of the DSF eye drops are shown in Table 1.

In Vitro Transcorneal Penetration of DDC from DSF Eye Drops  The in vitro transcorneal penetration of DDC from DSF eye drops was examined using the method of Iwata et al. Adult Japanese albino rabbits weighing 2.5 to 3.0 kg were killed by injecting a lethal dose of pentobarbital (0.6 mg/kg) 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 50 μl of 0.1, 0.25 or 0.5% DSF eye drops was instilled into the eyes of the rabbits. The aqueous humor samples (5 μl each) were removed from the anterior chamber of the eye for 0—90 min. The 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. 1:

\[ C_{DCC} = C_0 e^{-\lambda t} \]  

where \( C_{DCC} \) is the DDC concentration in the aqueous humor at time \( t \), \( C_0 \) is the initial concentration of DDC in the aqueous humor, and \( \lambda \) is the elimination rate constant of DDC from the aqueous humor. The \( C_{DCC} \) obtained in 5 experiments was 0.0502/min.

The DDC concentration data in the aqueous humor after the instillation of 50 μl of DSF eye drops were analyzed according to Eq. 2:

\[ C_{DCC} = \frac{k_F X}{V_a (k_a - k_e)} (e^{-k_a t} - e^{-k_e t}) \]  

where \( C_{DCC} \) is the DDC concentration in the aqueous humor, \( X \) is the dose of the DSF eye drop instillation, \( k_a \) is the absorption rate constant, \( V_a \) is the distribution volume (anterior chamber, ca. 150 μl), \( F \) is the fraction of DDC absorption, and \( \tau \) is the lag time. The area under the DDC concentration–time curve (\( AUC_{DCC} \)) was calculated according to the following equation (Eq. 3):

\[ AUC_{DCC} = \int_0^\infty C_{DCC} dt \]  

Briefly, \( AUC \) was determined according to the trapezoidal rule up to the last DDC concentration measurement point.

Measurement of Intraocular Pressure in Rabbits  The experiment was carried out according to Bonomi et al. The IOP in rabbits was measured with an electronic tonometer (Medtronic SOLAN, Jacksonville, FL, U.S.A.) under surface anesthesia (0.4% Benoxil). IOP elevation was induced by the rapid infusion of 5% glucose solution through the rabbit marginal ear vein. The amounts injected were 15 ml/kg of body weight and the infusion was accomplished in all animals within 20 s. The various eye drops were instilled 30 min prior to the infusion of the 5% glucose solution. The area under the curve (\( AUC_{IOP} \)) of the IOP (mmHg) versus time (min) (the area under IOP–time curve) was calculated as the difference of \( AUC \) in rabbits with or without the infusion of 5% glucose solution.

Table 1. Formulations of DSF Eye Drops

<table>
<thead>
<tr>
<th>Content (%)</th>
<th>DSF</th>
<th>HPβCD</th>
<th>HPMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1% DSF eye drops</td>
<td>0.10</td>
<td>1.1</td>
<td>0.1</td>
</tr>
<tr>
<td>0.25% DSF eye drops</td>
<td>0.25</td>
<td>3.0</td>
<td>0.1</td>
</tr>
<tr>
<td>0.5% DSF eye drops</td>
<td>0.50</td>
<td>5.0</td>
<td>0.1</td>
</tr>
</tbody>
</table>
results


glucose solution through the marginal ear vein. \( \Delta AUC_{\text{IOP}} \) was calculated according to the following equation (Eq. 4):

\[
\Delta AUC_{\text{IOP}} = AUC_{\text{IOP}} \text{ of saline instilled rabbit} - AUC_{\text{IOP}} \text{ of DSF eye drops instilled rabbit}
\]

**Measurement of NO Levels in the Aqueous Humor**

Adult Japanese albino rabbits weighing 2.5 to 3.0 kg were anesthetized by injecting pentobarbital (0.6 mg/kg) 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 with silicon tubing (inner diameter: 0.5 mm, Fuji Systems Co., Tokyo, Japan) was inserted into the eye to obtain aqueous humor samples, and 50 \( \mu l \) of DSF eye drops was instilled into the eyes. Aqueous humor samples (5 \( \mu l \) each) were collected from the anterior chamber of the eye, and the NO levels in the aqueous humor were measured using a Hitachi F-3000 Fluorescence Spectrophotometer (Hitachi, Tokyo, Japan) and NO2/NO3 Assay Kit-F II (Wako, Osaka, Japan) according to the manufacturer's instructions. In this paper, NO amounts reflect the levels of the NO2 and NO3 metabolites, which are the products of NO.

**Statistical Analysis**

All values are presented as mean±standard error of the mean (S.E.). Unpaired Student's or Aspin–Welch'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**

**Transcorneal Penetration of DDC from DSF Eye Drops**

Figure 1 shows *in vitro* transcorneal penetration of DDC from DSF eye drops using rabbit corneas. In this study, only DDC, not DSF, was detected. The amount of penetrated DDC increased after instillation of 0.1 or 0.25% DSF eye drops. There were no significant differences in the amount of penetration between the two DSF eye drop formulations. Figure 2 shows the DDC concentrations in the aqueous humor after the instillation of 0.1 or 0.25% DSF eye drops into rabbit eyes, and Table 2 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 at 20 min after the instillation of 0.25% DSF eye drops. In contrast to the *in vitro* transcorneal penetration results, the amount of DDC penetration for the 0.25% DSF eye drops was higher than that for the 0.1% eye drops, and the values of \( AUC_{\text{DDC}} \) and \( k_a \) for the 0.25% DSF eye drops were approximately 2.5-fold higher in comparison with those of the 0.1% DSF eye drops.

**Effect of DSF Eye Drops on IOP in Rabbit**

Figure 3 shows the effects of the instillation of DSF eye drops on IOP in rabbits with experimentally induced ocular hypertension.

**Table 2. Pharmacokinetic Parameters for the *in Vivo* Transcorneal Penetration of DDC Released from DSF Eye Drops**

<table>
<thead>
<tr>
<th>DSF Concentration</th>
<th>( AUC_{\text{DDC}} ) (( \mu M \cdot \text{min} ))</th>
<th>( k_a ) (min)</th>
<th>( \tau ) (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1% DSF eye drops</td>
<td>738±62</td>
<td>0.025±0.004</td>
<td>5.84±0.67</td>
</tr>
<tr>
<td>0.25% DSF eye drops</td>
<td>1835±313*</td>
<td>0.072±0.007*</td>
<td>4.99±0.22</td>
</tr>
</tbody>
</table>

\( AUC_{\text{DDC}}, \) the area under the DDC concentration–time curve; \( k_a, \) absorption rate constant; \( \tau, \) lag time. The data are presented as means±S.E. of 5 independent rabbits.

\( * p<0.05, \) vs. 0.1% DSF eye drops.

Fig. 2. *In Vivo* Transcorneal Penetration of DDC from DSF Eye Drops

Rabbits were instilled with 50 \( \mu l \) of 0.1 or 0.25% DSF eye drops. 0.1% DSF eye drop instilled rabbits (○), 0.25% DSF eye drop instilled rabbits (■). The data represent the means±S.E. of 5 rabbit corneas. ◆ \( p<0.05, \) vs. 0.1% DSF eye drops instilled rabbit.

Fig. 3. Effect of DSF Eye Drops on IOP in Rabbits Receiving a Rapid Infusion of 5% Glucose Solution

Rabbits receiving a rapid infusion of 5% glucose solution were instilled with 50 \( \mu l \) of saline or DSF eye drops. Saline instilled rabbits (○), 0.1% DSF eye drop instilled rabbits (■), 0.25% DSF eye drop instilled rabbits (●), 0.5% DSF eye drop instilled rabbits (□). The data represent as means±S.E. of 5 independent rabbits.
and Table 3 shows the IOP-reducing effect of DSF eye drops. The elevation of IOP in rabbits was induced by the rapid infusion of 5% glucose solution (15 ml/kg) through the marginal ear vein. The IOP was highest 10 min after the infusion, and returned to the pre-infusion level by 50 min after the infusion. Rabbits instilled with DSF eye drops showed a significantly reduced level of IOP elevation compared with rabbits instilled with saline, and the IOP-reducing effect increased with the increase in the DSF concentration of the eye drops (Table 3). Figure 4 shows the changes of NO levels in the aqueous humor of rabbits instilled with saline or 0.5% DSF eye drops. NO levels in the aqueous humor of rabbits rose following the rapid infusion of 5% glucose solution into the marginal ear vein, and reached a maximum at 20 min. The instillation of DSF eye drops also reduced the degree of NO elevation in the aqueous humor.

**DISCUSSION**

Glaucoma is a major cause of blindness, with an estimated 70 million people affected worldwide. Over the course of their lives, 10% of these patients will become bilaterally blind. However, for reasons of effectiveness and safety, a potent anti-glaucoma drug for human use has not yet been introduced. From the view point of the recent steep increase in the number of patients with glaucoma in modern aging societies, the development of effective and safe anti-glaucoma drugs is highly anticipated. In this study, we investigated the effects of DSF eye drops on IOP.

The safety of eye drops is an important factor. It has been reported that HPβCD ranks second in safety to γ-CD in a variety of CD derivatives used for eye drop applications. Moreover, Jansen et al. reported no irritation to eye membranes by HPβCD solutions less than 12.5%. Therefore, we used 1—5% HPβCD, which is low in comparison with the concentrations used in that report, and decided the DSF concentrations to be used in eye drops.

In both *in vitro* and *in vivo* transcorneal penetration experiments using rabbit corneas only DDC was detected in the reservoir side or aqueous humor. We previously reported a sulfhydryl-rich protein, aldehyde dehydrogenase 3A1 (ALDH3A1), which is related to the conversion of DSF to DDC, and exists in abundance in the corneal stroma and endothelium. Therefore, DSF in HPβCD solutions containing HPMC may be converted to DDC via catalysis by ALDH3A1 in the cornea. The amount of DDC penetration showed no significant difference between 0.1 and 0.25% DSF eye drops in the *in vitro* transcorneal penetration experiments (Fig. 1). In contrast, in the *in vivo* transcorneal penetration experiments, the amount of DDC penetration was about 4-fold higher in the case of the 0.25% DSF eye drops than the 0.1% DSF eye drops, and the values of AUC for 0.1% DSF eye drops were also higher for the 0.25% DSF eye drops than the 0.1% DSF eye drops (Fig. 2). In the *in vitro* transcorneal penetration experiments, the viscosity and corneal contact area of the 0.1 and 0.25% DSF eye drops were almost the same, since the DSF eye drops were completely stirred in the chamber. The results suggest that the absorption rate for transcorneal DDC penetration is regulated by the viscosity and corneal contact area of the DSF eye drops. Therefore, we measured the viscosity of two DSF drop preparations. The viscosity of the 0.25% DSF eye drops was higher than that of the 0.1% DSF eye drops (1.85 m²/s for the 0.1% DSF eye drops, 2.01 m²/s for the 0.25% DSF eye drops; measured at 25 °C by an Uberode type viscometer). It was known when the eye drops was lost from tear film within 30 s—2 min, while a small amount remained associated with conjunctival tissue. On the other hand, the increase in viscosity of the eye drops remained a higher storage on the surface of a cornea.

These suggest that the 0.25% DSF eye drops have a higher storage on the surface of a cornea, and this lead to the higher absorption rate for 0.25% DSF eye drops than the 0.1% DSF eye drops. In addition, free DSF and HPβCD-included DSF coexists both in 0.1 and 0.25% DSF solutions. Moreover, the concentrations of free DSF in those solutions are almost same. Therefore, the free DSF related strongly to the transcorneal penetration.

The intravenous administration of a 5% glucose solution is a simple and reproducible technique for the screening of anti-glaucoma agents. In this study, the IOP in rabbits receiving a rapid infusion of 5% glucose solution initially rose, and returned to baseline levels by 50 min after infusion. This indicates that the IOP elevation in this experiment may provide a suitable model for acute glaucoma. The degree of IOP elevation in rabbits receiving a rapid infusion of 5% glucose solution was reduced by the instillation of DSF eye drops with both 0.1 and 0.25% DSF eye drops providing IOP-reducing effects sufficient to protect against glaucoma. In addition, the IOP-reducing effect was greater for the higher DSF concentration (Table 3).

Table 3. The IOP-Reducing Effect of DSF Eye Drops in Rabbits Receiving Rapid Infusions with 5% Glucose Solution

<table>
<thead>
<tr>
<th>DSF Concentration</th>
<th>ΔAUCIOP (mmHg·min)</th>
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<tbody>
<tr>
<td>0.1% DSF eye drops</td>
<td>52.4 ± 4.9</td>
</tr>
<tr>
<td>0.25% DSF eye drops</td>
<td>61.8 ± 5.4</td>
</tr>
<tr>
<td>0.5% DSF eye drops</td>
<td>107.8 ± 14.7*</td>
</tr>
</tbody>
</table>

ΔAUCIOP was calculated as the difference of AUC IOP of saline instilled rabbit and ΔAUCIOP of DSF eye drops instilled rabbit. The data represent the mean ± S.E. of 5 independent rabbits. *p < 0.05, vs. 0.1% DSF eye drops.

![Fig. 4. Effect of DSF Eye Drops on NO in Rabbit Rapid Infused with 5% Glucose Solution](image)

The rabbits rapid infused with 5% glucose solution were instilled with 50 μl of saline or 0.5% DSF eye drops. Saline instilled rabbit (○), 0.5% DSF eye drops instilled rabbit (●). The data represent the means ± S.E. of 5 independent rabbits. *p < 0.05, vs. saline instilled rabbit.
It is important to clarify the mechanism by which DSF eye drops protect against the elevation in IOP. Shah et al. reported that the rapid infusion of a 5% glucose solution into rabbits leads to a reduction in blood osmolarity, which leads to the transfer of water into the eye thus causing the elevation in IOP. Kiel et al. reported that the inhibition of NO by t-NAME causes a decrease in water production by due to ciliary vasoconstriction in rabbits, resulting in a decrease in IOP. In addition, it was known that the IOP elevation in rabbits receiving a rapid infusion of 5% glucose solution used this study was caused by rapidly aqueous humor production. In our previous study, we reported that DSF eye drops prevent excess NO production in the eye. We used 0.5% DSF eye drops to clarify the IOP-reducing mechanism of DSF eye drops, because the higher concentration makes it easier to clarify the IOP-reducing effects. NO levels in the aqueous humor of rabbits receiving a rapid infusion of 5% glucose solution increased, and the instillation of 0.5% DSF eye drops reduced this increase (Fig. 4). The instillation of DSF eye drops did not affect the IOP of normal rabbit (without the rapid infusion of 5% glucose solution, data not shown). Taking these findings together, we hypothesize that the DSF eye drops penetrate the cornea, and that DSF is converted in the cornea to DDC by ALDH3A1. This DDC may cause a reduction in water production by inhibiting NO production, resulting in a reduction in IOP. On the other hand, the elevation of IOP occurs prior to the induction of NO in the aqueous humor. In this study, the NO amounts reflect the levels of the NO\textsuperscript{a} and NO\textsuperscript{1} metabolites, which are the products of NO. Therefore, the high NO levels by 20 min after the infusion may show metabolites of NO. As incipient NO produced in the eye may be consumed for the ciliary vasoconstriction, the apparent enhancement of NO in the aqueous humor may not change.

Further studies are needed to elucidate the precise mechanisms of the IOP-reducing effect of DSF eye drops. Therefore, we are now investigating the effect of DSF eye drops on aqueous production.

In conclusion, the present study demonstrates that IOP and NO levels increase in rabbits receiving a rapid infusion of 5% glucose solution, and the instillation of DSF eye drops reduces these increases. The instillation of DSF eye drops has a potent IOP-reducing effect in rabbits with experimentally induced high IOP, probably by inhibiting the elevation in NO levels. These findings provide significant information that can be used in designing further studies to develop antiglaucoma drugs.

REFERENCES

1) Armaly M. F., Krueger D. E., Maunder L., Becker B., Hetherington J.


17) Kristinsson J. K., Fridriksdottir H., Thorisdottir S., Sigurdardottir A., 1578 Vol. 33, No. 9


25) Kristinsson J. K., Fridriksdottir H., Thorisdottir S., Sigurdardottir A., 1578 Vol. 33, No. 9


