

Regular Article

A Nanoparticle-Based Ophthalmic Formulation of Dexamethasone Enhances Corneal Permeability of the Drug and Prolongs Its Corneal Residence Time

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We designed ophthalmic formulations containing dexamethasone-loaded solid nanoparticles (DEX_{nano} dispersion), and investigated corneal permeability and toxicity. 0.1% dexamethasone (DEX) powder (DEX microparticles), 0.026% methyl *p*-hydroxybenzoate (MP), 0.014% propyl *p*-hydroxybenzoate (PP), and 0.5% methylcellulose were used, and the DEX_{nano} dispersion was prepared by the bead mill method. The mean particle size of DEX_{nano} dispersion was 78 nm. Antimicrobial activity of the DEX_{nano} dispersion were measured by using *Escherichia coli*, and the corneal epithelium-debrided rat model and HCE-T cells (immortalized human corneal epithelial cell line) were used to estimate the corneal toxicity. The transcorneal penetration of the DEX_{nano} dispersion were evaluated in the corneas of rabbit. The DEX_{nano} dispersion was found to be highly stable until 14 d after its preparation. Although DEX itself did not exhibit antimicrobial activity, the DEX_{nano} dispersion containing parabens (MP and PP) showed high antimicrobial activity, approximately equal to that of the solution containing parabens without DEX. The corneal penetration rate (*J*_c) and mean residence time (*MRT*) of DEX from the DEX_{nano} dispersion were approximately 5.1- and 1.3-fold higher, respectively, than those of a dispersion containing DEX microparticles (mean particle size, 11.3 μm). In addition, no significant difference was found in corneal stimulation between the vehicle and DEX_{nano} dispersion. In conclusion, we successfully prepared high quality dispersion containing DEX solid nanoparticles, and the nanoparticle-based ophthalmic formulation of DEX enhanced the corneal permeability and residence time of the drug. It is possible that DEX_{nano} dispersion will show increased effectiveness in treating ocular inflammation.

Key words nanoparticle; dexamethasone; corneal permeability; eye drop; drug delivery system

Topically applied dexamethasone (DEX), a corticosteroid, is used in the treatment of ocular inflammation, such as in uveitis and cystoid macular edema related to cataract surgery.¹⁾ DEX permeates biological membranes quite easily, since it is relatively lipophilic. However, in the ophthalmic field, its low solubility (0.16 mg/mL in water) limits its clinical usefulness.²⁾ Owing to its poor solubility, it is formulated as aqueous solutions of water-soluble derivatives, such as DEX sodium phosphate and DEX metasulfobenzoate sodium. However, DEX formulated as a solution of its water-soluble salt has low corneal permeability due to the poor partitioning of the hydrophilic DEX derivative into the lipophilic corneal epithelium, which works as the protective barrier for the ocular system.³⁾ Ophthalmic DEX ointments that lengthen the residence times of the dose instilled and enhance ophthalmic bioavailability are also used in the management and prevention of ocular inflammation.⁴⁾ However, ophthalmic ointments have not been used extensively because of drawbacks such as blurred vision and low patient compliance.⁵⁾ Thus, there is a pressing need for ophthalmic delivery systems that combine high solubility and corneal permeability.

To improve these problems, the usefulness of an ophthalmic drug system using viscous solutions, hydrogels, and micro/nanoparticles has recently been studied.^{6–12)} Numerous studies

have found that viscous solutions do not possess sufficient mechanical strength to resist the ocular clearance mechanism, and offer only a transient improvement in ocular residence time.¹³⁾ On the other hand, it has been reported that the nanoparticles is possible to penetrate across the membrane.^{6–10)} The biodegradable polymer poly(DL-lactide-co-glycolide) (PLGA) has been widely utilized as a carrier for bioactive molecules and presents a possible solution to the limitations regarding ocular drug penetration (mean particle diameter, 50–200 nm).^{6,7,14–18)} We have also reported the method of drug nanoparticles using the bead mill,^{19–25)} and showed that dispersions containing tranilast, indomethacin, or cilostazol nanoparticles enhanced corneal penetration as compared with traditional formulations (drug solutions type, eye drops).^{20–23)} We hypothesized that an ophthalmic formulation of DEX nanoparticles prepared using the bead mill method might offer high corneal permeability, and that enhancing transcorneal penetration of DEX would increase its effectiveness in treating ocular inflammation (as can occur in uveitis and after cataract surgery), and lead to an expansion of its usage as a therapy in the ophthalmologic field.

In this study, we designed new ophthalmic formulations containing DEX solid nanoparticles, and demonstrated the effect of these ophthalmic formulations on corneal permeability. In addition, we investigated the toxicity, antimicrobial, and

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activity stability of ophthalmic formulations containing DEX solid nanoparticles.

MATERIALS AND METHODS

Animals and Materials All experiments were performed in accordance with the Use of Laboratory Animals, and the Association for Research in Vision and Ophthalmology resolution on the use of animals in research and the Kindai University Faculty of Pharmacy Committee Guidelines for the Care. Japanese albino rabbits (2.5–3.0 kg, Shimizu Laboratory Supplies Co., Ltd., Kyoto, Japan) and Wistar rats (7 weeks, Kiwa Laboratory Animals Co., Ltd., Wakayama, Japan) were used in this study. All animals were housed under controlled lighting condition (7:00–19:00 h/19:00–7:00 h, fluorescent light/dark). Dexamethasone powder (solid, DEX microparticles; particle size (mean±standard deviation (S.D.)), 11.3±0.314 μm), methyl *p*-hydroxybenzoate (MP) and propyl *p*-hydroxybenzoate (PP) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). A commercially available 0.1% dexamethasone metasulfobenzoate sodium eye drop solution (Santeson[®] ophthalmic solution 0.1%, CA-DEX eye drops) was provided by Santen Pharmaceutical Co., Ltd. (Osaka, Japan). SM-4 methylcellulose (MC) was obtained from Shin-Etsu Chemical Co., Ltd. (Tokyo, Japan). All other chemicals used were of the highest purity commercially available.

Preparation of Ophthalmic Formulations Containing DEX Nanoparticles The preparation of nanoparticles was performed by using the zirconia beads (diameter: 0.1, 2 mm), Bead Smash 12 (Wakenyaku Co., Ltd., Kyoto, Japan) according to our previous reports.²⁰⁾ The DEX powder containing 2 mm zirconia beads were treated with the Bead Smash 12 for 30 s (3000 rpm, 4°C), and was dispersed in saline, and crushed again with the Bead Smash 12 (5500 rpm, 30 s×15 times, 4°C) using 0.1 mm zirconia beads. The compositions of the dispersion containing DEX are shown in Table 1. The pH in the ophthalmic dispersions containing 0.1% (2.5 mm) DEX microparticles (DEX_{micro} dispersion) and nanoparticles (DEX_{nano} dispersion) was adjusted 6.8. The SALD-7100 (Shimadzu Corp., Kyoto, Japan) and SPM-9700 (Shimadzu Corp.) were used to measure the particle sizes (refractive index 1.60–0.10i) and images, respectively.

Stability of Ophthalmic Formulations Containing DEX Nanoparticles The experiment was performed according to our previous reports.^{20,22)} Ophthalmic dispersions (3 mL) containing DEX were incubated in 5 mL test tubes in the dark at 20°C for 14 d, after which 50 μL sample was withdrawn from above 8–10% part. The concentrations of DEX were determined using Shimadzu LC-20AT system (Shimadzu Corp.) at 254 nm (HPLC method). The 10 μg/mL butyl *p*-hydroxybenzoate, 50 mM phosphate buffer (pH 6.8)–acetonitrile (70:30, v/v) were used as internal standard and mobile phase, and the

sample and internal standard was injected into an Inertsil[®] ODS-3 column (GL Science Co., Inc., Tokyo, Japan) at a flow rate of 0.25 mL/min (35°C).

Antimicrobial Activity of Ophthalmic Formulations Containing DEX The experiment was performed according to our previous reports using *Escherichia coli* (*E. coli*, ATCC 8739).²⁰⁾ The *E. coli* (1 organism per aliquot) was incubated in the presence of saline containing 0.5% MC (MC solution); saline containing 0.026% MP and 0.014% PP (Paraben solution); MC solution plus 0.1% DEX nanoparticles (DEX_{nano} without parabens); or DEX_{nano} dispersion (with parabens) at 20 to 25°C. The data was represented as log colony-forming units (CFU) values.

In Vitro Transcorneal Penetration of Ophthalmic Formulations Containing DEX The experiment was performed according to our previous reports using a methacrylate cell designed for transcorneal penetration studies.^{20,22)} The donor chamber exposed to the exterior surface of the cornea was filled with ophthalmic dispersion containing DEX. The reservoir chamber was filled with 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) buffer (pH 7.4) containing 136.2 mM sodium chloride, 5.3 mM potassium chloride, 1.0 mM dipotassium phosphate, 1.7 mM calcium chloride and 5.5 mM glucose. The experiments were performed at 35°C over the course of 6 h. Fifty microliters of sample solution were withdrawn from the reservoir chamber at the indicated time intervals and replaced with the same volume of buffer. DEX concentrations were determined by HPLC as described above. The obtained data were analyzed using the following equations (Eqs. 1–3):

$$\tau = \frac{\delta^2}{6D} \quad (1)$$

$$J_c = \frac{K_m \cdot D \cdot C_{\text{DEX}}}{\delta} = K_p \cdot C_{\text{DEX}} \quad (2)$$

$$Q_t = J_c \cdot A \cdot (t - \tau) \quad (3)$$

where C_{DEX} is the DEX concentration in DEX ophthalmic dispersion; τ is the lag time; δ is the thickness of the cornea (0.0625 cm); J_c is the DEX penetration rate; K_p is the penetration coefficient through the cornea; K_m is the cornea/preparation partition coefficient; Q_t is the total amount of DEX appearing in the reservoir solution at time t ; A is the effective area of the cornea (0.78 cm²); and D is the diffusion constant within the cornea.^{20,22)} In this study, the viability of the corneas was monitored by measurements of thickness or weight.

In Vivo Transcorneal Penetration of Ophthalmic Formulations Containing DEX The experiment was performed according to the method reported previously by us.^{20,22)} Forty microliters of dispersion containing DEX was instilled into the eyes of the rabbits, and 5 μL aqueous humor samples were collected periodically for 90 min. DEX concentrations were

Table 1. Changes in DEX Particle Size in DEX_{micro} and DEX_{nano} Dispersions 14 d after Treatment with a Bead Mill

Formulation	Content (w/v%)				Treatment	Particle size (μm)	
	DEX microparticles	MP	PP	MC		Immediately	14 d after preparation
DEX _{micro} dispersion	0.1	0.026	0.014	0.5	—	11.3±0.31	11.8±0.33
DEX _{nano} dispersion	0.1	0.026	0.014	0.5	Bead mill	0.078±0.059	0.073±0.060

DEX particle sizes of dispersion containing DEX were determined by a nanoparticle size analyzer SALD-7100 (refractive index 1.60–0.10i). Means±S.D.

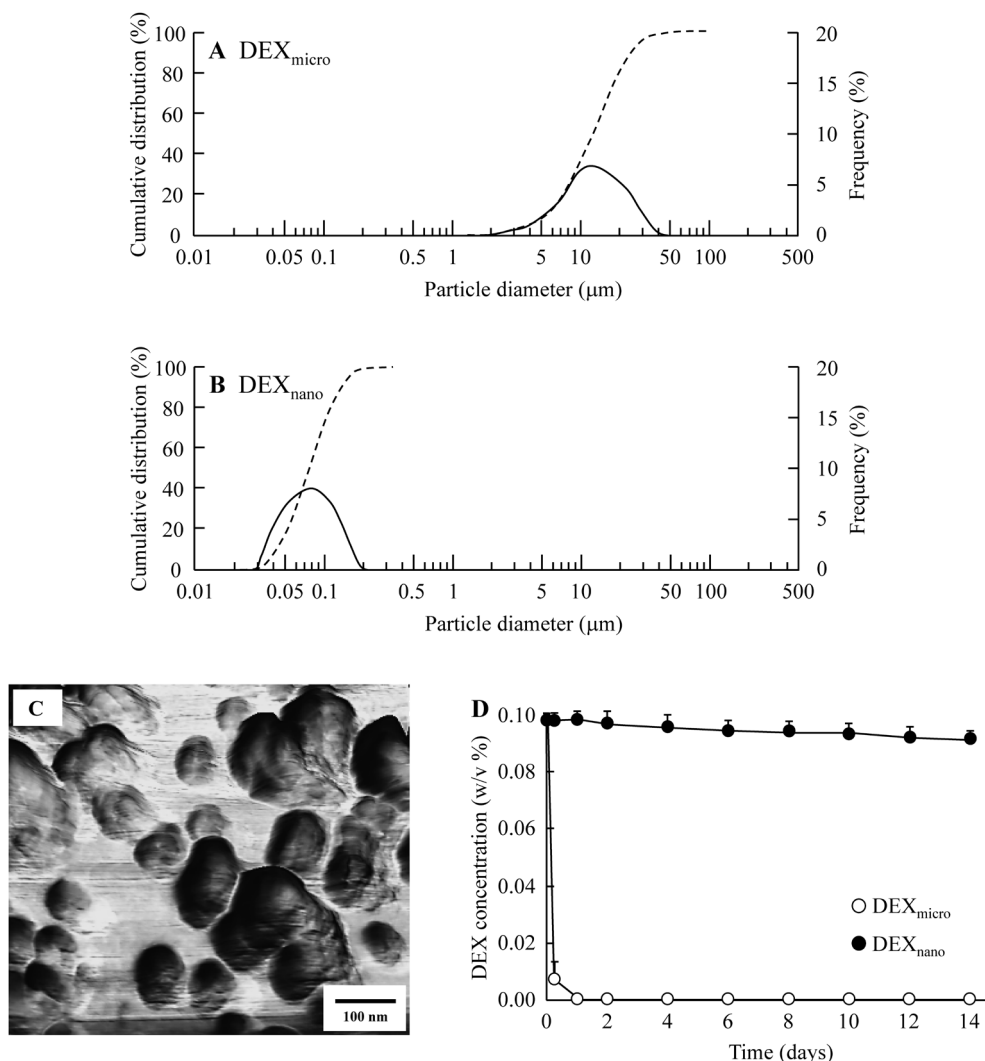


Fig. 1. Cumulative Size Distribution, Frequency, Images and Stability of a Nanoparticle-Based Ophthalmic Formulation of Dexamethasone (DEX)

The compositions of the ophthalmic dispersion containing DEX are shown in Table 1. A and B: Cumulative size distribution (dashed line) and frequency (solid line) of DEX_{micro} (A) and DEX_{nano} (B) dispersion. C: Image of the DEX_{nano} dispersion. D: Stability of the DEX_{nano} dispersion. 0.1% DEX_{micro} (○) and 0.1% DEX_{nano} (●) dispersions were kept in the dark at 20°C for 14 d. Mean ± S.E., $n=8$.

determined by HPLC as described above. The area under the DEX concentration–time curve ($AUC_{0-90\text{ min}}$) and the mean residence time (MRT) were calculated according to the following equations (Eqs. 4–6):

$$AUC_{0-90\text{ min}} = \int_0^{90} C_{\text{DEX}} dt \quad (4)$$

$$AUMC_{0-90\text{ min}} = \int_0^{90} C_{\text{DEX}} \cdot t dt \quad (5)$$

$$MRT = \frac{AUMC_{0-90\text{ min}}}{AUC_{0-90\text{ min}}} \quad (6)$$

C_{DEX} is the DEX concentration at time t after eye drop instillation (0–90 min).

Image Analysis of Corneal Wound Healing in Rats Instilled with Dispersions Containing DEX Nanoparticles

The experiment was performed according to our previous reports using 1% fluorescein (Alcon Japan, Tokyo, Japan) and a TRC-50X fundus camera (Topcon, Tokyo, Japan).^{20,22} A patch of corneal epithelium was removed with a BD Micro-Sharp™ (blade 3.5 mm, 30°; Becton Dickinson, Fukushima, Japan). The areas of debrided corneal epithelium were as

follows: saline, $12.17 \pm 0.51 \text{ mm}^2$; vehicle in DEX dispersion, $11.90 \pm 0.48 \text{ mm}^2$; DEX_{micro} dispersion, $12.45 \pm 0.61 \text{ mm}^2$; and DEX_{nano} dispersion, $12.31 \pm 0.66 \text{ mm}^2$ (mean ± standard error (S.E.) for eight independent rat corneas). Forty microliters of eye drops were instilled at 9:00, 12:00, 15:00, 18:00, and 21:00 (five times per day). The percentage of corneal wound healing and the corneal wound healing rate constant (k_H , h^{-1}) were calculated according to Eqs. 7 and 8:

$$\begin{aligned} \text{Corneal wound healing (\%)} \\ = (\text{wound area}_{0\text{ h}} - \text{wound area}_{12-36\text{ h}}) \\ / \text{wound area}_{0\text{ h}} \times 100 \end{aligned} \quad (7)$$

$$H_t = H_\infty \cdot (1 - e^{-k_H t}) \quad (8)$$

H_∞ and H_t are the percentages of corneal wound healing (%) at time ∞ and t (0–36 h) after corneal abrasion, respectively.

Cell Culture and Treatment The experiment was performed according to our previous reports using HCE-T cells (immortalized human corneal epithelial cell line) and Cell Count Reagent SF (Nacalai Tesque Inc., Kyoto, Japan).^{20,22} 1×10^4 HCE-T cells were seeded in 96-well microplates

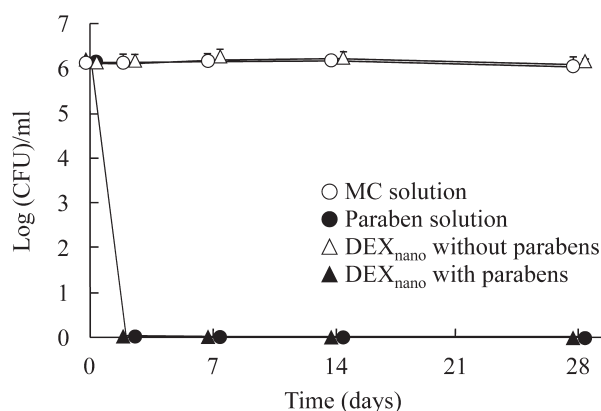


Fig. 2. Evaluation of Antimicrobial Activities of a Nanoparticle-Based Ophthalmic Formulation of DEX

Saline containing 0.5% MC (○, MC solution), saline containing 0.026% MP and 0.014% PP (●, Paraben solution), MC solution plus 0.1% DEX nanoparticles (△, DEX_{nano} without parabens), and MC solution plus paraben solution and 0.1% DEX nanoparticles (▲, DEX_{nano} with parabens) were tested for antimicrobial activity against *E. coli*. Mean $\pm$ S.E., $n=5$.

(IWAKI, Chiba, Japan). Saline, vehicle solution, DEX_{micro} dispersion, or DEX_{nano} dispersion was added to the cell cultures one day after seeding, and the cells were stimulated for 0–10 min. The stimulation time was determined according to the *in vivo* retention time of drugs in the cornea (about 2 min).^{7,8,26} The cell viability (%) was calculated as ratio ($\text{Abs}_{\text{treatment}}/\text{Abs}_{\text{non-treatment}} \times 100$).

Measurement of Intraocular Pressure in Rabbits Forty microliters of ophthalmic dispersions containing DEX as described in Table 1 were instilled into the right eyes of the rabbits at 10:00 a.m. (once a day) for four weeks. Intraocular pressure (IOP) was measured with an electronic tonometer (Medtronic SOLAN, Jacksonville, FL, U.S.A.) under surface anesthesia (0.4% Benoxil).

Statistical Analysis Statistical comparisons were performed using the unpaired Student's *t*-test or Dunnett's multiple comparison using JMP (SAS Institute Inc., Cary, NC, U.S.A.). $p < 0.05$ was considered statistically significant.

RESULTS

Design of Ophthalmic Dispersion Containing DEX Nanoparticles Figures 1A–C show the particle size distributions of ophthalmic dispersions containing DEX. DEX microparticles ($11.3 \pm 0.314 \mu\text{m}$) containing MP and PP reached a meringue state when milled using the bead mill, whereas it was possible to mill DEX microparticles containing MP, PP, and MC using the bead mill method to a mean particle size of $78 \pm 59 \text{ nm}$ (mean $\pm$ S.D.; DEX_{nano} dispersion). Figure 1D shows the stability of DEX_{micro} and DEX_{nano} dispersions. The DEX_{micro} dispersion precipitated completely by 3 h after preparation. The stability of ophthalmic dispersion containing DEX was enhanced by using both an additive mixture (MP, PP and MC) and the bead mill method, and precipitation of the DEX_{nano} dispersion was not observed until 14 d after preparation. Figure 2 shows the antimicrobial activity of ophthalmic dispersions containing DEX. The DEX_{nano} dispersion without parabens (MP and PP) did not exhibit antimicrobial activity; however, the DEX_{nano} dispersion containing MP and PP showed high antimicrobial activity, which was approximately

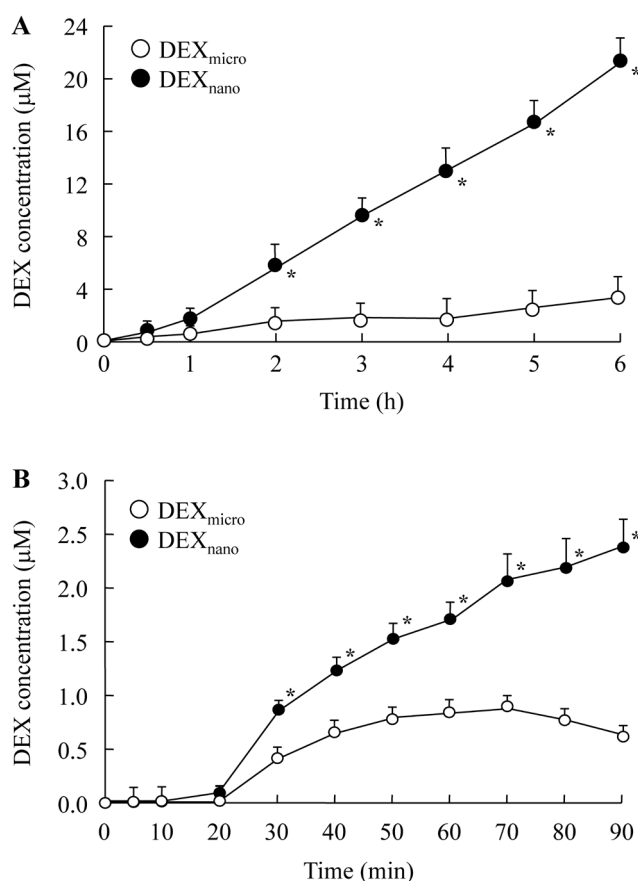


Fig. 3. *In Vitro* and *In Vivo* Transcorneal Penetration of a Nanoparticle-Based Ophthalmic Formulation of DEX

The compositions of the ophthalmic dispersion containing DEX are shown in Table 1. A: *In vitro* transcorneal penetration of DEX_{micro} and DEX_{nano} dispersion. The donor chamber exposed to the exterior surface of the cornea was filled with 0.1% DEX_{micro} (○) or 0.1% DEX_{nano} (●) dispersion. B: *In vivo* transcorneal penetration of DEX_{micro} and DEX_{nano} dispersion. Rabbit eyes were instilled with 40 μL of 0.1% DEX_{micro} (○) or 0.1% DEX_{nano} (●) dispersion. Mean $\pm$ S.E., $n=7$. * $p < 0.05$, vs. DEX_{micro} dispersion-instilled rabbit.

equal to that of a paraben (MP and PP) solution.

Corneal Permeability of Ophthalmic Dispersion Containing DEX Nanoparticles Figure 3A shows the *in vitro* transcorneal penetration of DEX_{micro} and DEX_{nano} dispersions through rabbit corneas, and *in vitro* study in Table 2 summarizes the pharmacokinetic parameters calculated from the *in vitro* transcorneal penetration data. The transcorneal penetration was increased linearly for 6 h, and no significant changes in thickness or weight were observed at the 6 h period. The J_c , K_p , K_m and D of the DEX_{nano} dispersion were significantly higher, and the τ for DEX_{nano} dispersion was lower than for the DEX_{micro} dispersion. Figure 3B shows the *in vivo* transcorneal penetration of DEX_{micro} and DEX_{nano} dispersion through rabbit corneas, and *in vivo* study in Table 2 summarizes the pharmacokinetic parameters calculated from the *in vivo* transcorneal penetration data. The DEX concentration in the aqueous humor after the instillation of the DEX_{micro} or DEX_{nano} dispersion was detected. The transcorneal penetration in the case of DEX_{micro} dispersion began after a lag time of 21.4 min, and the lag time from DEX_{nano} dispersion was 18.1 min. In addition, the *MRT* value for DEX_{nano} dispersion was significantly higher than that of the DEX_{micro} dispersion, and the $AUC_{0-90 \text{ min}}$ value for the DEX_{nano} dispersion was approximately 2.25 times greater than that of the DEX_{micro} dispersion.

Table 2. Pharmacokinetic Parameters for the *in Vitro* and *in Vivo* Transcorneal Penetration of the Nanoparticle-Based Ophthalmic Formulation of DEX

<i>In vitro</i> study	J_c (pmol·cm ⁻² ·min ⁻¹)	K_p (×10 ⁻⁶ ·min ⁻¹)	K_m (×10 ⁻⁵)	τ (min)	D (×10 ⁻⁴ ·cm ² ·min ⁻¹)
DEX _{micro} dispersion	39±4	1.8±0.2	8.6±0.1	48.9±2.1	1.34±0.25
DEX _{nano} dispersion	200±39*	11.0±2.3*	29.0±4.7*	33.6±5.6*	3.42±1.03*
<i>In vivo</i> study	$AUC_{0-90\text{min}}$ (mm·min)	Lag time (min)	MRT (min)		
DEX _{micro} dispersion	48.6±5.1	21.4±0.97	52.1±1.35		
DEX _{nano} dispersion	109.9±9.6*	18.1±0.89*	67.5±1.97*		

Parameters were calculated according to Eqs. 1–6 (see Materials and Methods). The compositions of the ophthalmic dispersion containing DEX are shown in Table 1. Means±S.E., $n=7$. * $p<0.05$, vs. DEX_{micro} dispersion for each category.

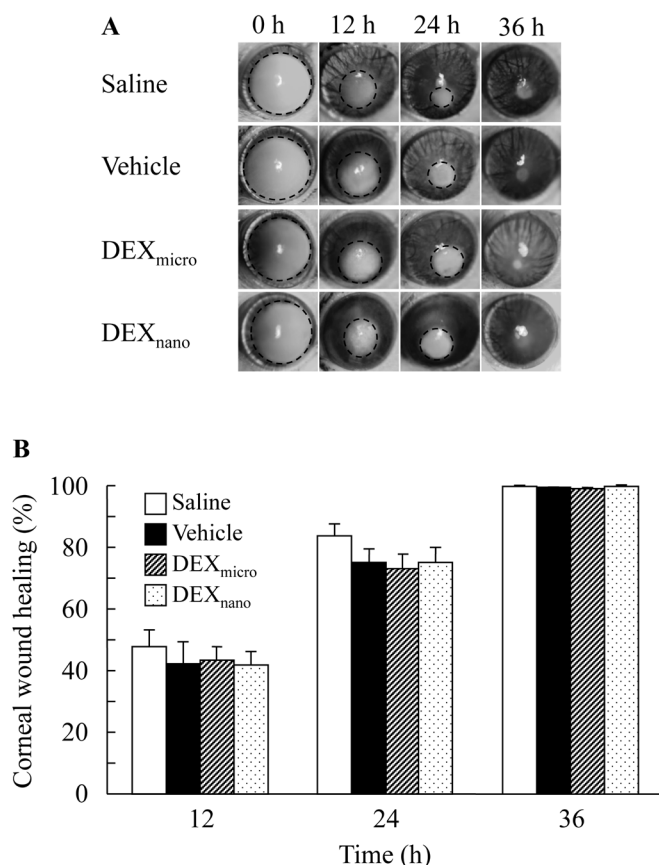


Fig. 4. Corneal Wound Healing of Rat Eyes with or without the Instillation of a Nanoparticle-Based Ophthalmic Formulation of DEX

Corneal images (A) and corneal wound healing (B) of rat eyes treated by instilling DEX_{micro} or DEX_{nano} dispersion (five times per day). The inside of the dashed lines shows the corneal wound. Rats were instilled with Saline (saline). Rats instilled with vehicle in DEX dispersion (vehicle), 0.1% DEX_{micro} dispersion-instilled rats (DEX_{micro}), or 0.1% DEX_{nano} dispersion-instilled rats (DEX_{nano}). The compositions of the ophthalmic dispersion containing DEX are shown in Table 1. Mean±S.E., $n=8$.

Evaluation of Safety in the Instillation of Ophthalmic Dispersion Containing DEX Nanoparticles Figure 4 shows images after corneal epithelial abrasion (A), and levels of corneal wound healing (B) following the instillation of DEX_{micro} and DEX_{nano} dispersions. The levels of corneal wound healing of rat eyes instilled with saline was approximately 47.8% at 12h, and the levels of corneal wound healing at 24h was 83.3%. At 36h after corneal epithelial abrasion, the corneal wounds of rat eyes instilled with saline had almost entirely healed. The corneal wounds of rat eyes instilled with the vehicle showed 72.5% healing 24h after corneal

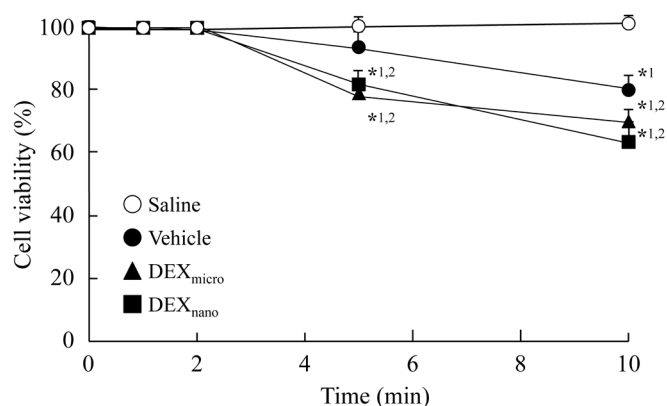


Fig. 5. Effects of a Nanoparticle-Based Ophthalmic Formulation of DEX on the Viability of HCE-T Cells

HCE-T cells in 96-well microplates were treated with saline (○, saline), vehicle in DEX dispersion (●, vehicle), 0.1% DEX_{micro} dispersion (▲, DEX_{micro}), or 0.1% DEX_{nano} dispersion (■, DEX_{nano}) for 0–10 min. The compositions of the ophthalmic dispersion containing DEX are shown in Table 1. Mean±S.E., $n=8-10$. * $p<0.05$, vs. saline for each category; * $p<0.05$, vs. vehicle for each category.

epithelial abrasion, and the k_H of rat eyes instilled with the vehicle (4.63 ± 0.56 , $\times 10^{-2}$ /h, mean±S.E., $n=8$) was a little lower than that of eyes instilled with saline (5.26 ± 0.66 , $\times 10^{-2}$ /h, mean±S.E., $n=8$). Contrarily, no significant difference was found in the k_H between saline and the vehicle. The corneal wounds of rat eyes instilled with DEX_{micro} and DEX_{nano} dispersions showed 73.2 and 75.1% healing 24h after corneal epithelial abrasion, respectively, and the k_H of both DEX_{micro} (4.55 ± 0.51 , $\times 10^{-2}$ /h) and DEX_{nano} (4.57 ± 0.54 , $\times 10^{-2}$ /h) dispersions was similar to that of the vehicle (mean±S.E., $n=8$). Figure 5 shows changes in the viability of HCE-T cells following treatment with DEX_{micro} and DEX_{nano} dispersions. The viability of HCE-T cells treated with vehicle, DEX_{micro}, and DEX_{nano} dispersions was almost the same as that of those treated with saline for 0–2 min, and cell stimulation was not observed. Contrarily, after DEX treatment for 5 min and 10 min, the viability of HCE-T cells treated with DEX_{nano} dispersion decreased, to 81.0 and 64.2%, respectively. The viability of HCE-T cells treated with the DEX_{nano} dispersion was similar to that of those treated with the DEX_{micro} dispersion. Figure 6 shows the effects of the DEX_{micro} and DEX_{nano} dispersions on IOP in rabbits. The IOP in the normal rabbit was 13.3 ± 1.8 mmHg, and this remained unchanged with the continuous instillation of DEX_{micro} and DEX_{nano} dispersions over four weeks.

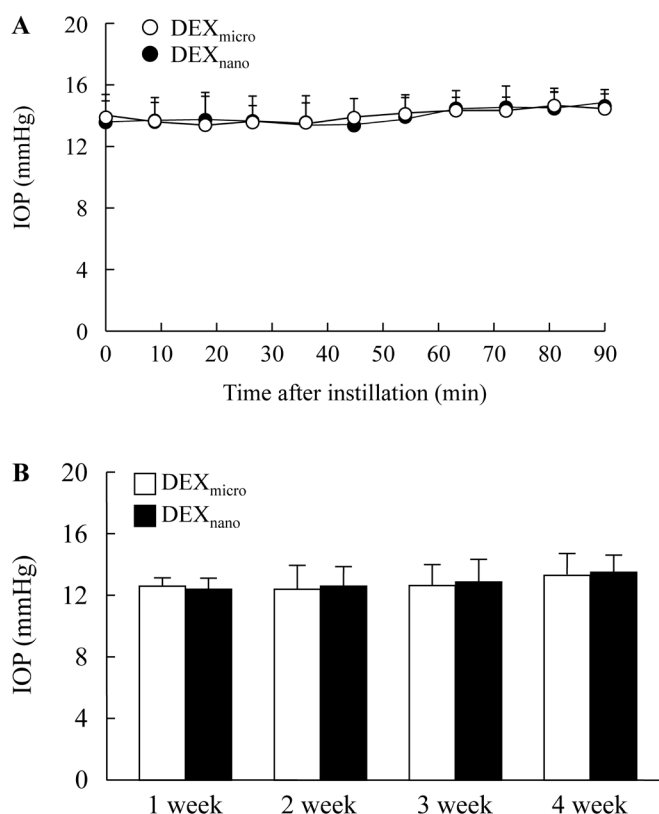


Fig. 6. Effect of a Nanoparticle-Based Ophthalmic Formulation of DEX on IOP in Rabbits

The compositions of the ophthalmic dispersion containing DEX are shown in Table 1. A: Changes in IOP levels in rabbit 0–90 min after instillation of 0.1% DEX_{micro} (○) or 0.1% DEX_{nano} (●). B: Changes in IOP levels in rabbit continuously instilled with DEX dispersion for 1–4 weeks. Rabbits were instilled with 0.1% DEX_{micro} dispersion-instilled rabbits (□, DEX_{micro}) or 0.1% DEX_{nano} dispersion-instilled rabbits (■, DEX_{nano}) into the right eye once a day (10:00 a.m.) for four weeks. Mean ± S.E., $n=8$.

DISCUSSION

Topically applied DEX is used in the treatment of ocular inflammation; however, the clinical use of its most commonly marketed eye drop formulations is limited because DEX has low aqueous solubility, which means that a water-soluble derivative (DEX sodium phosphate or DEX metasulfobenzoate sodium) must be used. However, there is poor partitioning of hydrophilic DEX derivatives into the cornea. Thus, there have been efforts to develop novel formulations that can be used as eye drops and have high bioavailability. Recently, the nanoparticle-based ophthalmic drug systems are expected to lead about improvements in terms of reducing the side-effects of drug therapies in the field of ophthalmology.^{15,16,22} In this study, we designed new ophthalmic formulations containing DEX solid nanoparticles (DEX_{nano} dispersion), and investigated their usefulness in ophthalmology by evaluating their stability, antimicrobial activity, corneal permeability, and toxicity.

The selection of additives is important to design the ophthalmic dispersions containing DEX solid nanoparticles by bead mill. We previously reported that the addition of MC, highly biocompatible, is indispensable to the preparation of nanoparticles using the bead mill method,^{19–22,24,25} and the MC is already used in the preparation of ophthalmic formulations.^{27–29} Therefore, we selected MC as an additive in this

study. Preservatives are also usually added to pharmaceutical products to prevent decomposition due to the actions of bacteria. Among preservatives, benzalkonium chloride (BAC) and parabens, such as MP and PP, are commonly used in the preparation of eye drops. Although BAC has a stronger preservative effect than parabens, its corneal toxicity is greater.^{30,31} Furthermore, parabens are already used as preservatives in commercially available DEX eye drops (Santeson® ophthalmic solution 0.1%, CA-DEX eye drops). Based on this research, we attempted to prepare a DEX dispersion containing MP, PP, and MC using the bead mill method (particle size of DEX_{nano} dispersion without parabens (mean ± S.D.), 79 ± 60 nm). Just as was previously reported,^{19–22,24,25} the bead mill method without MC led to the meringue-like state; however, the addition of MC improved the meringue-like state, and DEX particle size was reached <100 nm by the bead mill treatment used DEX microparticles, MP, PP and MC (DEX_{nano}, Fig. 1). It is expected that DEX_{nano} dispersion may provide an ophthalmic delivery systems that high corneal permeability.

Next, we examined whether the stability (Fig. 1D) and preservative effect (Fig. 2) of DEX change in DEX_{nano} dispersion. At 14 d after preparation, the DEX_{nano} dispersion showed highly stable (Fig. 1D), and the stability was remained for 1 month; the stability of DEX_{nano} formulation was 0.076 ± 0.007% (w/v) at 1 month after the preparation (particle size 93 ± 114 nm, $n=5$). Moreover, we confirmed whether the DEX nanoparticles affect the antimicrobial activity by parabens in this study, since the eye drops containing solid nanoparticles was novel formulation. The DEX_{nano} dispersion containing parabens showed high antimicrobial activity, and the levels in antimicrobial activity was approximately equal to that of the paraben solution alone (Fig. 2). In addition, HPLC methods did not show degradation or reduction of DEX in the DEX dispersion (concentration of DEX_{nano} dispersion without parabens 14 d after preparation, 0.1%, $n=8$). These results suggest that the DEX nanoparticles in the dispersion prepared in this study did not affect the antimicrobial activity of the parabens, and that there is no difference in chemical structure between DEX microparticles and nanoparticles.

Furthermore, we evaluated the transcorneal penetration of DEX_{nano} dispersion. The corneal penetration and MRT of DEX observed in the DEX_{nano} dispersion were significantly higher than those observed in the DEX_{micro} dispersion (Table 2). In the ophthalmic field, it has been reported that nanoparticles, sizes <100 nm, facilitate improved topical passage of large, water insoluble molecules through the barriers of the ocular system.³² We also reported that an nanoparticle-based ophthalmic formulation enhanced the K_p , K_m , and D in comparison with those observed when microparticles were used, resulting in increased drug transcorneal penetration.^{20,22,23} In this study, the particle size in the DEX_{nano} dispersion (78 nm) was lower than 100 nm, and the K_p , K_m , and D of the DEX_{nano} dispersion were all significantly higher than those in the DEX_{micro} dispersion. The J_c was also enhanced. Moreover, the τ for the DEX_{nano} dispersion was lower than that for the DEX_{micro} dispersion (Table 2). Based on these results, it could be suggested that the nano order size of the solid DEX may be the reason that transcorneal penetration is enhanced through the improvement of K_p , K_m , and D . In addition, the J_c and MRT of the DEX_{nano} dispersion were both significantly higher than that of CA-DEX eye drops (J_c (mean ± S.E.), 34 ± 5 pmol/cm²/min;

MRT (mean $\pm$ S.E.), 40.3 $\pm$ 1.01 min; $n=6$). The CA-DEX eye drops contained the hydrophilic DEX derivative³⁾; therefore, the poor partitioning into the lipophilic epithelium may be related to its low transcorneal penetration and residence time.

It is important to elucidate the toxicity of the DEX_{nano} dispersion. We previously reported that the experimental methods using cultured cell and rat model debrided corneal epithelium can evaluate the slight corneal toxicity of eye drops.²²⁾ Therefore, we used these experimental methods in this study. The viability of HCE-T cells treated with DEX_{nano} dispersion was almost the same as that of those treated with saline or vehicle for 0–2 min (Fig. 5), and no significant difference was found in the k_H between the vehicle, DEX_{micro} and DEX_{nano} dispersions. Moreover, abnormal findings were not observed in rabbit corneas when the instillation of 0.1% DEX_{nano} dispersion (40 μ L) was continued for four weeks (once a day, 10:00 a.m.). In addition, we compared the corneal stimulation of the DEX_{nano} dispersion and the CA-DEX eye drops. The viability of HCE-T cells and k_H of rat eyes treated with the DEX_{nano} dispersion was higher than that of those treated with the CA-DEX eye drops (viability of HCE-T cells for 2 min (mean $\pm$ S.E.), 92.6 $\pm$ 0.9%, $n=8$; k_H (mean $\pm$ S.E.), 3.61 $\pm$ 0.40, $\times 10^{-2}$ /h, $n=8$). These results show that the nanoparticle-based ophthalmic formulation reduces the corneal toxicity of DEX eye drops, and that the corneal stimulation effect of DEX_{nano} dispersion is lower than that of the CA-DEX eye drops, which have various additives, such as a solubilizing agent and surface active agent.

On the other hand, it was known that continuous instillation and intravitreal injection of DEX increased the IOP (a side-effect of DEX), so we investigated the effect of the DEX_{nano} dispersion on IOP. The IOP was not changed by the continuous instillation of DEX_{micro} and DEX_{nano} dispersions over four weeks (Fig. 6). From these results, we conclude that the instillation of DEX_{nano} dispersion does not have a significant influence on IOP. Further studies are needed to elucidate the anti-inflammatory effect of DEX_{nano} dispersion using *in vivo* model, such as endotoxin-induced uveitis rats.

In the present study, we succeeded in preparing high quality dispersion containing DEX solid nanoparticles (mean particle size, 78 nm), and the corneal penetration and *MRT* of DEX from the DEX_{nano} dispersion were significantly higher than those of the CA-DEX eye drops. It is possible that DEX_{nano} dispersion will show increased effectiveness in treating ocular inflammation.

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Conflict of Interest The authors declare no conflict of interest.

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