TABLE 2. Interaction of 4 Combinations Using LVFX Against Keratitis Isolates

Organism (No. Isolates)		FIC Index Mean ± SD	FIC Index Range	Rate of Additivity (%)	Rate of Indifference (%)
Staphylococcus species (22)	LVFX + CMX	1.05 ± 0.48	0.563–2	82	18
	LVFX + TOB	1.38 ± 0.59	0.508–2	55	45
	LVFX + EM	1.91 ± 0.29	1.0–2.0	9	91
	LVFX + CP	1.95 ± 0.21	1.0–2.0	5	95
Streptococcus species (5)	LVFX + CMX	1.05 ± 0.33	0.75–2	80	20
	LVFX + TOB	1.73 ± 0.61	0.625–2	20	80
	LVFX + EM	1.35 ± 0.60	0.75–2	60	40
	LVFX + CP	1.20 ± 0.45	1.0–2.0	80	20
Gram-negative rods (7)	LVFX + CMX	1.04 ± 0.44	0.75–2	86	14
	LVFX + TOB	1.04 ± 046	0.531–2	86	14
	LVFX + EM	1.20 ± 0.57	0.625–2.0	71	29
	LVFX + CP	1.17 ± 0.59	0.75–2.0	71	29

Epidemiological surveys revealed that bacterial keratitis is predominantly caused by *Staphylococcus* species, *Streptococcus* species, or gram-negative rods (chiefly *P. aeruginosa*).[1,2] Clinical findings are often variable, depending on causative bacteria; for example, keratitis caused by gram-positive cocci, including *Staphylococcus* species and *Streptococcus* species, is associated with well-defined grayish white infiltrates and localized ulcerations, whereas keratitis caused by gram-negative rods, including *P. aeruginosa*, is associated with hazy corneal rings and soupy ulcerations. Therefore, possible causative bacteria can be inferred at the initial visit in most cases.[13,14] In addition, corneal scraping smear examination may provide early recognition of possible causative bacteria. If causative bacteria can be recognized, however, there are insufficient criteria for determining whether an antibacterial eye drop, to which causative bacteria may exhibit high sensitivity, would be used alone or in combination.

In the present study, we investigated the combined activities of LVFX and nonfluoroquinolone antibacterial eye drops commercially available in Japan (CMX, TOB, CP, and EM) and found that quinolone + CMX combination showed low FIC index against both *Staphylococcus* species and *Streptococcus* species. Quinolone + CMX combination also had the lowest FIC index against gram-negative rods and exhibited additive activity against isolates at high rate. Additive activity of LVFX + TOB was observed in 55% of isolates of *Staphylococcus* species, whereas EM and CP showed poor interaction with LVFX against *Staphylococcus* species, and these combinations were considered to result in no increased activity. FIC indexes of LVFX + CP and LVFX + EM were lower than those of LVFX + TOB against *Streptococcus* species. These findings suggest that when infection with gram-positive cocci (staphylococci or streptococci) is suspected, fluoroquinolone + CMX combination may be effective. However, against gram-negative rods, additive activities against isolates were observed at high rates, 70% or higher, for all drug combinations tested, and especially, favorable FIC indexes were obtained with LVFX + TOB and LVFX + CMX.

In experiment 1, favorable FIC indexes were obtained with LVFX + CMX against gram-positive cocci and with LVFX + TOB against gram-negative rods. We therefore investigated whether the combined activities were different among quinolones in experiment 2. Against gram-positive cocci, quinolone + CMX, compared with quinolone + TOB, had lower FIC indexes and higher rates of additive activity. Among quinolone + TOB combinations, LVFX + TOB had the most favorable FIC indexes, and among quinolone + CMX combinations, LVFX or GFLX + CMX had lower FIC indexes and higher rates of additive activity compared with MFLX + CMX.

According to MIC90 data of each drug alone or in combination, MIC90 of GFLX or MFLX was reduced to 1/2 when combined with CMX, and MIC90 of CMX was reduced to 1/4 when combined with GFLX or LVFX. These findings

TABLE 3. Interaction of 6 Combinations Against 35 Gram-Positive Cocci Isolates

	FIC Index Mean ± SD	FIC Index Range	Rate of Additivity (%)	Rate of Indifference (%)
LVFX + TOB	1.35 ± 0.63	0.516–2	54	46
GFLX + TOB	1.51 ± 0.58	0.625–2	43	57
MFLX + TOB	1.57 ± 0.58	0.563–2	37	63
LVFX + CMX	1.07 ± 0.53	0.531–2	77	23
GFLX + CMX	1.11 ± 0.55	0.516–2	74	26
MFLX + CMX	1.31 ± 0.59	0.531–2	60	40

TABLE 4. Interaction of 6 Combinations Against 12 Gram-Negative Rod Isolates

	FIC Index Mean ± SD	FIC Index Range	Rate of Additivity (%)	Rate of Indifference (%)
LVFX + TOB	1.31 ± 0.62	0.625–2	58	42
GFLX + TOB	1.49 ± 0.64	0.625–2	42	58
MFLX + TOB	1.59 ± 0.56	0.75–2	33	67
LVFX + CMX	1.19 ± 0.50	0.75–2	75	25
GFLX + CMX	0.96 ± 0.37	0.502–2	92	8
MFLX + CMX	0.89 ± 0.38	0.502–2	92	8

TABLE 5. MIC90 of Tested Isolates With or Without Combination

		MIC90 (mg/L)			
		Fluoroquinolone Without Combination	**Fluoroquinolone With Combination**	**TOB or CMX Without Combination**	**TOB or CMX With Combination**
Gram-positive cocci	LVFX + TOB	LVFX:128	LVFX:32	TOB:16	TOB:16
	GFLX + TOB	GFLX:64	GFLX:64	TOB:16	TOB:8
	MFLX + TOB	MFLX:64	MFLX:32	TOB:16	TOB:16
	LVFX + CMX	LVFX:128	LVFX:128	CMX:8	CMX:2
	GFLX + CMX	GFLX:64	GFLX:32	CMX:8	CMX:2
	MFLX + CMX	MFLX:64	MFLX:32	CMX:8	CMX:4
Gram-negative rod	LVFX + TOB	LVFX:1	LVFX:0.5	TOB:0.5	TOB:0.5
	GFLX + TOB	GFLX:2	GFLX:1	TOB:0.5	TOB:0.5
	MFLX + TOB	MFLX:4	MFLX:4	TOB:0.5	TOB:0.5
	LVFX + CMX	LVFX:1	LVFX:0.5	CMX:16	CMX:16
	GFLX + CMX	GFLX:2	GFLX:0.5	CMX:16	CMX:8
	MFLX + CMX	MFLX:4	MFLX:1	CMX:16	CMX:8

may indicate differences in combined activity among quinolones. Especially against gram-positive cocci, CMX combined with LVFX or GFLX had the lowest MIC, which was 1/64 to 1/4 of MICs of fluoroquinolone alone or in combination and CMX alone. Therefore, when infection with gram-positive cocci is suspected, it is preferable to combine CMX with LVFX or GFLX for increasing activity of CMX. Meanwhile, against gram-negative rods, FIC indexes of fluoroquinolone + CMX combinations were lower than those of fluoroquinolone + TOB. MIC90 values of CMX in combination were, however, higher than those of TOB alone. Against gram-negative rods, MIC90 values were not different between TOB alone and in combination with quinolone, whereas MIC90 values of LVFX and GFLX were reduced to 1/2 when combined with TOB. The lowest MIC90 was obtained with a combination of LVFX + TOB, which had a favorable MIC90 of 0.5 in combination. These findings suggested that antibacterial activity of LVFX and GFLX against gram-negative rods may be increased when combined with TOB. Our experiments, although they were in vitro, suggested that some combinations may increase antibacterial activity of each drug used. Some studies have suggested that in vitro susceptibility may predict clinical outcome in bacterial keratitis.[15–17] Thus, compared with monotherapy with an antibacterial eye drop, combination therapy with 2 drugs that have beneficial interaction may increase antibacterial activity, resulting in improved treatment effect.

The combined activity of fluoroquinolone + additional antimicrobial was previously reported.[18–21] Especially against *P. aeruginosa*, a causative bacterium of keratitis, synergy was observed between GFLX and cefepime or gentamicin. Similar interaction, although smaller, was observed in our experiments.[21] Sueke et al[9] investigated combined activity against 7 isolates of *S. aureus* and 5 isolates of *P. aeruginosa* from keratitis using E-test. They reported that meropenem + ciprofloxacin had additive or synergistic activities against *S. aureus* and *P. aeruginosa* and that teicoplanin + meropenem, ciplofloxacin, or moxifloxacin had additive or synergistic activities against *S. aureus*. Thus, β-lactam and teicoplanin, which inhibit cell wall synthesis, may have beneficial interaction with fluoroquinolone. As eye drops, CMX and cephalosporin may be of choice.

The present study suggests that combination of fluoroquinolone with CMX or TOB may be a feasible option for improving effects of fluoroquinolone and taking measures against fluoroquinolone-resistant bacteria. Although cost of combination therapy must be considered, future investigations are needed on the possibility of cost reduction because of early induction of healing. In addition, because of the difference in combined activity among fluoroquinolones, drugs should be selected depending on causative bacteria.

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In Vitro Efficacy of Ocular Surface Lubricants Against Dehydration

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Purpose: To compare 3 ocular lubricants containing sodium hyaluronate (SH), carboxymethylcellulose (CMC), and hydroxypropyl methylcellulose (HPMC) for their ability to enhance water retention and to protect human corneal epithelial cells (HCECs) from dehydration.

Methods: Experiments were performed using 0.1% and 0.3% solutions of the 3 lubricants diluted in Milli-Q water for the water retention assays and in DMEM/F12 culture medium for the cell viability assays. Five milliliters of each of the lubricants was dropped onto a filter paper, and the paper was kept in an open container at 25°C and a humidity of 36% to 38%. The weight of the paper was measured hourly for 4 hours. In the second set of experiment, cultured HCECs were exposed to the test lubricants for 60 minutes, and the lubricants were removed. Cells were then exposed to room air for up to 60 minutes. Cells were then incubated with a vital dye, and the absorption of the reduced form of the dye was measured. The cell survival rate was compared for the 3 lubricants.

Results: Filter papers moistened with both 0.1% and 0.3% SH were significantly heavier than those moistened with CMC and HPMC at all time points. The survival rate of HCECs was significantly higher at most times with 0.1% and 0.3% SH than with CMC and HPMC solutions. The effects of CMC were not significantly different from those of HPMC.

Conclusions: These findings indicate that SH is significantly better for water retention and protection of HCECs from dehydration than HPMC and CMC.

Key Words: lubricant, tear, corneal epithelial cell, dry eye disease, dehydration

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Dry eye disease is a relatively common ailment affecting approximately 5% to 30% of the adult population.[1] It is a multifactorial disease of the tears and ocular surface that results in discomfort, visual disturbances, and tear film instability with the potential of damaging the ocular surface.[2] Artificial tears are the mainstay treatment of dry eye disease. Although there are many effective artificial tear formulations, sodium hyaluronate (SH), carboxymethylcellulose (CMC), and hydroxypropyl methylcellulose (HPMC) are the main components of artificial tears most commonly prescribed.

Investigations have been performed on the effectiveness of these lubricants in protecting the viability of corneal epithelial cells in a porcine dry eye model,[3] in improving the tear film stability in a rabbit model,[4] and in treating mild-to-moderate dry eyes in humans.[5] However, at present, there has been no single report simultaneously comparing these 3 lubricants for their ability to enhance water retention and to protect cultured human corneal epithelial cells (HCECs) against dehydration.

Thus, the aim of this study was to compare SH, CMC, and HPMC lubricating solutions for their ability to retain water and to enhance the viability of HCECs against dehydration. To accomplish this, we selected concentrations of 0.1% and 0.3% for the 3 solutions as is used in most artificial tear preparations and performed 2 sets of experiments on water retention and protection of HCECs against dehydration.

MATERIALS AND METHODS

Lubricants

Sodium hyaluronate (SH; Kewpie, Tokyo, Japan), carboxymethylcellulose (CMC; Dai-ichi Kogyo Seiyaku Co, Ltd, Tokyo, Japan), and hydroxypropyl methylcellulose (HPMC; Shin-Etsu Chemical Co, Ltd, Tokyo, Japan) were purchased and prepared at 0.1% and 0.3% concentrations. The lubricants were dissolved in Milli-Q water for the water retention assays and in DMEM/F12 culture medium (Nacalai Tesque, Kyoto, Japan) for cell viability assays.

Human Corneal Epithelial Cells

SV-40 immortalized HCEC line was purchased from RIKEN BioResource Center (Ibaraki, Japan) for the in vitro studies. Cells were incubated in DMEM/F12 culture medium with 15% fetal bovine serum, 5 μg/mL of insulin, 10 ng/mL of human EGF, and 40 μg/mL of gentamicin in cell culture flasks at 37°C in an atmosphere of 5% CO_2 in air.

Evaluation of Water Retention

Five milliliters of each lubricant solution was dropped onto a standard filter paper (Filter Paper Quantitative 90 mm; diameter, 90 mm; thickness, 0.21 mm; Toyo Roshi Kaisha, Ltd, Tokyo, Japan). The lubricant-soaked filter paper was

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immediately weighed with a semimicro balance (Sartorius, Tokyo, Japan), and the weight was set as the value at time 0. All samples were kept at room temperature (25°C) and constant humidity (36%–38%) in an open container throughout the study. The soaked filter papers were measured hourly for 4 hours, and each lubricant was tested for 6 times. Filter papers soaked with Milli-Q water only were similarly measured and served as control.

Evaluation of Protective Efficacies of Different Lubricants on HCECs Against Dehydration

In the second set of experiments, the 3 lubricants were tested for their ability to prevent the death of HCECs exposed to dehydrating conditions. For this study, 1.0×10^4 HCECs/100 μL DMEM/F12 were cultured in a medium containing 40 μg/mL of gentamicin per well in 96-well plates (Corning Incorporated, NY). The cells were cultured at 37°C in an atmosphere of 5% CO_2 in air overnight. The supernatant was removed, and the cells were rinsed with fresh DMEM/F12. Then, 50 μL of 0.1% or 0.3% of SH, CMC, or HPMC solutions was added to the cells and incubated for 1 hour at 37°C in an atmosphere of 5% CO_2 in air. After removal of the test solutions, the cells were dried in room air for 10, 20, 30, 45, and 60 minutes. Then, the cells were incubated for 2 hours at 37°C with DMEM/F12 culture medium containing a tetrazolium compound (CellTiter 96 Aqueous One Solution Cell Proliferation Assay; Promega Corporation, WI). The level of reduced dye, an indicator of metabolic activity, in the cells was measured with a microplate reader (Model 3550; Bio-Rad Laboratories, CA) at 490 nm according to the manufacturer's instructions. The survival rate of the cultured cells was calculated according to the following formula:

$$\text{Cell survival rate} = (\text{absorbance of lubricant pretreated} / \text{absorbance of negative control}) \times 100\%.$$

The negative controls were the cells that were not dehydrated (100% cell survival rate), and positive controls were the cells pretreated with DMEM/F12 culture medium only and dried in the same way. Each lubricant from 6 wells was tested, and the average was used for the statistical analyses.

Statistical Analyses

All data are expressed as the means ± SEM. Wilcoxon tests, Tukey variant tests, or Dunnett multiple tests were used to determine the significance of the differences in the water retention and cell survival rate. A $P < 0.05$ was taken to be significant.

RESULTS

Comparison of Water Retention Capacity

Immediately after dropping the lubricants, the filter papers soaked in 0.1% SH, 0.3% SH, and 0.3% CMC were significantly heavier than those of the controls that were moistened with Milli-Q water only ($P < 0.001$, $P < 0.001$, and $P = 0.003$, respectively, Tukey variant test). The weight of the SH-moistened filter papers was significantly heavier than that of those moistened with corresponding concentrations of CMC and HPMC (all Ps < 0.001; Figs. 1A, B). The differences between CMC and HPMC were not significant except at 2 hours where the filter papers wetted with 0.3% CMC were significantly heavier than those wetted with HPMC ($P = 0.000$; Fig. 1B). In addition, SH-soaked filter papers were significantly heavier than the controls at all times, and the differences in the weights were concentration dependent (all Ps = 0.000, Dunnett multiple test; Fig. 1C). The concentration-dependent effect was also noted for the first 3 hours with filter papers soaked with CMC (Fig. 1D) and only at 0 hours with papers soaked with HPMC (Fig. 1E).

Protection on HCECs Against Dehydration

HCECs were pretreated with the different concentrations of the different lubricants and dried for the selected time periods. For wells that were not exposed to dehydration (negative control), all of the cells (100%) survived for the 60-minute test period (Fig. 2). The average survival rates for cells exposed to DMEM/F12 culture medium alone (positive control) were 86.1%, 33.7%, 3.6%, 3.4%, and 2.6% at 10, 20, 30, 45, and 60 minutes, respectively. The average survival rates of cells pretreated with 0.1% SH were 100%, 76.1%, 19.2%, 5.2%, and 5.3% at each corresponding times. The survival rate of 0.1% SH-pretreated cells was significantly higher than that of 0.1% CMC-pretreated cells at 20 minutes ($P = 0.0025$; Tukey variant test) and also significantly higher than that of 0.1% HPMC-treated cells at 30, 45, and 60 minutes (all Ps < 0.05, Fig. 2A).

The 0.3% SH solutions had a significantly higher protective effect than CMC and HPMC after 20, 30, 45, and 60 minutes of dehydration ($P < 0.05$ at 20 and 30 minutes vs. CMC; Tukey variant test; $P < 0.05$ at 30, 45, and 60 minutes vs. HPMC). The cells pretreated with 0.1% CMC had a significantly higher survival rate at 30, 45, and 60 minutes than HPMC ($P < 0.05$). However, for 0.3% solutions, the difference between CMC and HPMC was not significant at all times (Fig. 2B). SH solutions had a dose-dependent effect in protecting cells from dehydration (Fig. 2C). In contrast, the survival rates of cells pretreated with CMC and HPMC were not different from those of positive controls, and except for CMC and HPMC at 20 minutes, no dose-dependent effect was noted for these 2 lubricants (Figs. 2D, E).

DISCUSSION

Commercially available SH, CMC, and HPMC solutions are the major lubricants used to treat the patients with dry eye. The high-viscosity polymer, CMC, was introduced into artificial tear preparations more than 60 years ago to retard canalicular drainage, which improved the contact time of the lubricant on the ocular surface. CMC is an anionic cellulose polymer with a carboxylic group. It is available in several viscosities, and the viscosity depends on the molecular weight. CMC has bioadhesive properties, and its anionic characteristic probably increases the tear retention time.

SH is a glycosaminoglycan disaccharide biopolymer and consists of repeating and alternating linear chains of

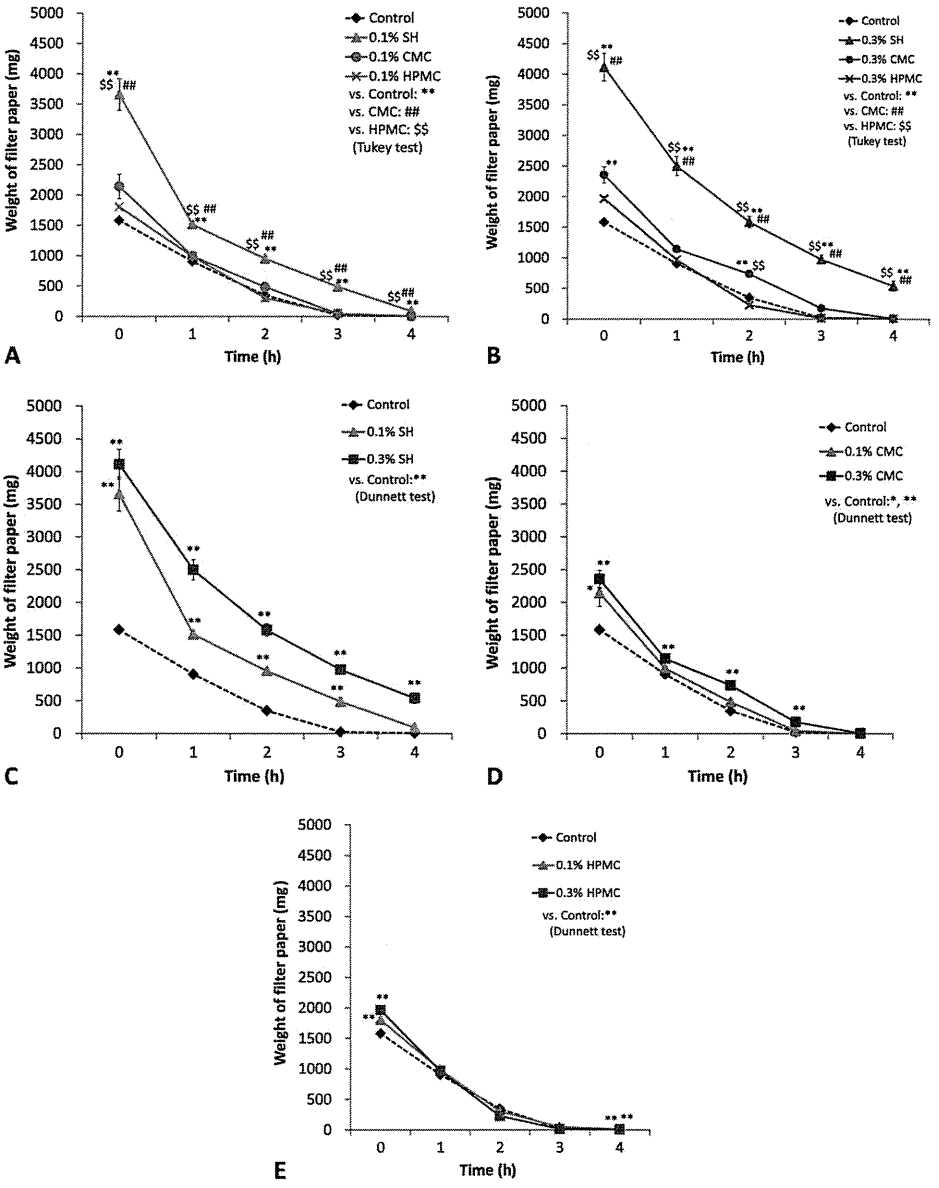

FIGURE 1. Water retention ability of 3 ocular surface lubricants: sodium hyaluronate (SH), carboxymethylcellulose (CMC), and hydroxypropyl methylcellulose (HPMC). Standard filter papers were soaked with 5 mL of each of the lubricants and left in an open container at 25°C and a humidity of 36% to 38%. The weight of the filter papers was measured hourly for 4 hours. Comparisons of 0.1% (A) and 0.3% (B) solutions are shown. The SH-moistened filter papers are significantly heavier at all time points than controls moistened with Milli-Q water in a concentration-dependent manner (C). The concentration-dependent effect was also noted for the first 3 hours with filter papers soaked with CMC (D) and only at 0 hours with papers soaked with HPMC (E). Data represent the means ± SEM of 6 samples. *, $P < 0.05$; **, $P < 0.01$ vs. control; ##, $P < 0.01$ vs. CMC; $$, $P < 0.01$ vs. HPMC. Analyzed by Tukey variant tests or Dunnett multiple tests as indicated.

N-acetylglucosamine and glucuronate. It has a large capacity to bind with water with an affinity of 1000-fold of its weight, and it is resistant to desiccation.

HPMC forms the polymeric component of several commercial tear substitutes. It is less viscous than CMC but has superior cohesive and emollient properties. Although the effectiveness of SH, CMC, or HPMC has been studied in a variety of experimental and clinical conditions, the lubricant which would be best for a specific patient has not been determined. In general, the choice is determined empirically or is based on the preference of the physician. To date, evidence from comparative studies of SH, CMC, and HPMC on their ability to retain water and to prevent the death of HCECs during dehydration has not been published.

We tested the water retention ability of the 3 lubricants by measuring the weight of filter papers dampened with the different lubricants. Our results showed that for both 0.1% and 0.3% solutions, the weight of SH-soaked filter papers was significantly heavier than that of those soaked in CMC and HPMC at almost all time points evaluated. This indicates that there must have been less evaporation of water from the filter paper soaked with the SH solution. These data are in agreement with the report of Nakamura et al[6] who demonstrated that SH can retard water loss from a solution or when placed on top of agar gel.

SH has a high molecular weight and is a naturally occurring glycosaminoglycan. It is a long flexible molecule with an open coil conformation.[7] The water retention ability of SH may be attributed to the special sponge-like structure of the polysaccharide chains. This property is also thought to contribute to the maintenance of tear film stability and its ability to keep the ocular surface moistened.[8]

Our findings also support the data reported by Snibson et al[9] that the ocular surface residence time of SH was

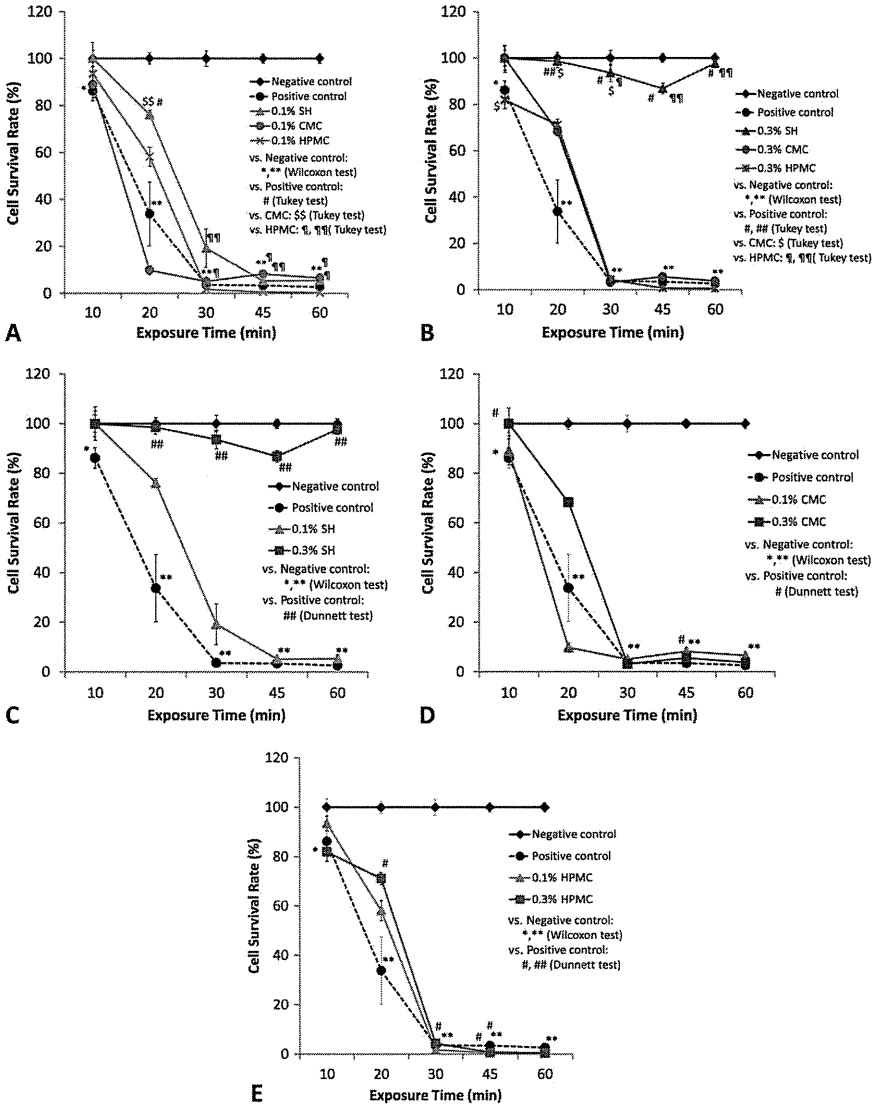

FIGURE 2. Protective effect of different ocular surface lubricants, SH, CMC, and HPMC, on HCECs from dehydration. Cells were exposed to the test lubricants for 1 hour and placed under dehydrating conditions. The survival rate of the cells was calculated and compared. The negative controls were cells not dried (100% survival rate), and positive controls were cells pretreated with culture medium only and dried similarly. Comparisons of 0.1% (A) and 0.3% (B) solutions are shown. In a dose-dependent manner, SH has a significantly higher protective effect at all times (C). Except for CMC and HPMC at 20 minutes, no dose-dependent effect was noted for these 2 lubricants (D and E). Data represent the means ± SEMs of 6 samples. *, $P < 0.05$; **, $P < 0.01$ vs. negative control; #, $P < 0.05$; ##, $P < 0.01$ vs. positive control; $, $P < 0.05$; $$, $P < 0.01$ vs. CMC; ¶, $P < 0.05$ ¶¶, $P < 0.01$ vs. HPMC. Analyzed by Wilcoxon tests, Tukey variant tests, or Dunnett multiple tests as indicated.

significantly longer than that of HPMC. Thus, SH could play a role of not only retaining water but also acting as a reservoir of slowly released water molecules, which would make this lubricant more suitable for artificial tear preparations.

The second set of our experiments was designated to evaluate the protective capability of the 3 lubricants against dehydration of HCECs. Again, our results demonstrated that for most of the time points evaluated, the HCECs survival rate was significantly higher for cells pretreated with SH than for those pretreated with CMC and HPMC. The effectiveness of SH was dose dependent. These data support the reports that SH treatment was effective in healing corneal lesions and in alleviating keratitis faster than CMC.[10] SH was also shown to increase the corneal surface regularity index better than CMC.[11]

The survival rates of cells pretreated with CMC and HPMC were not significantly different from their positive controls, indicating their ineffectiveness in protecting cultured corneal epithelial cells against dehydration. For some time points evaluated, for example, 0.1% CMC at 20 minutes, the cell survival rate was even lower than that of positive control, indicating the possibility of cytotoxicity of the lubricant. However, the difference was not statistically significant, and the changes were not consistent for both 0.1% and 0.3% solutions, that is, no dose-dependent effect. In addition, the standard error of the means was large at these time points. Thus, the low cell survival rate may reflect data variation rather than the cytotoxicity of the lubricant.

Beside its effectiveness in enhancing water retention and preventing dehydration, the clinical efficacy of SH in treatment of dry eye disease could also be attributed to the fact that SH can reduce the inflammatory response caused by dehydration. This is supported by a study that showed that SH was able to reduce the inflammatory marker CD44, a hyaluronate receptor that is known to be overexpressed in dry eye patients.[10] SH has also been shown to be beneficial for corneal epithelial migration and elongation in vitro.[12,13] These effects would therefore favor the growth of corneal epithelial cells in culture and contribute to the significantly

higher survival rate of cells pretreated with SH during dehydration.

Our data suggest that SH is superior to the 2 other lubricants tested for its water retention ability and its protective effectiveness of HCECs against dehydration. However, the active ingredient is not the only important factor of the best artificial tear preparation but the concentration, molecular weight, tonicity, preservatives, and other ionic components need to be considered. In addition, extrapolation of these data to their performance under dynamic conditions of the human ocular surface needs further studies in animal models and human subjects. Research on factors such as the dynamics of blinking and its effect on eye evaporation are also needed.

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New method for evaluation of early phase tear clearance by anterior segment optical coherence tomography

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ABSTRACT.

Purpose: **To describe a new method of measuring early phase tear clearance by anterior segment optical coherence tomography (AS-OCT).**

Methods: **Sixty normal subjects were divided into a young group (30 subjects; 29.6 ± 7.2 years) and an elder group (30 subjects; 71.4 ± 10.8 years). AS-OCT (CASIA SS-1000, Tomey, Japan) with customized software was used to record the tear meniscus at the centre of the lower eyelid. Five microlitres of lukewarm saline solution was dropped into the lower conjunctival sac, and an image of the tear meniscus was obtained immediately and again 30 seconds after natural blinking. The tear meniscus height (TMH) and tear meniscus area (TMA) were measured in the AS-OCT images, and the percentage decrease in the TMH and TMA was used as a measure of the tear clearance. Correlations between tear clearance and clinical features including degree of conjunctivochalasis, degree of protrusion of inferior lacrimal punctum, distance of lacrimal punctum from the Marx line and fluorescein clearance rates were also determined in another healthy population consisting of 30 subjects.**

Results: **The OCT tear clearance rate was 35.2 ± 11% for TMH and 28.1 ± 12.4% for TMA in the young group, and 12.4 ± 7.3% and 6.2 ± 9.1%, respectively in the elder group. The differences were significant for both the TMH ($p = 0.017$) and the TMA ($p = 0.024$). The OCT-determined tear clearance was positively correlated with the fluorescein clearance rate, and negatively correlated with the distance between the lacrimal punctum and Marx line, degree of conjunctivochalasis and degree of lacrimal punctum protrusion.**

Conclusion: **AS-OCT can be used as a rapid, non-invasive and quantitative method of determining the early phase tear clearance rate in a normal healthy population.**

Key words: anterior segment optical coherence tomography – lacrimal drainage – tear clearance – tear meniscus

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Introduction

Healthy tear clearance depends on the integrity of the lacrimal system, and the rate of clearance is the summation of tear secretion by the lacrimal glands and ocular surface epithelia, fluid transudation through the conjunctiva, tear evaporation, tear drainage through the nasolacrimal system, and corneal and conjunctival permeability (Paiva & Pflugfelder 2004; Tomlinson & Khanal 2005). Evidence from animal and human studies has shown that reduced tear clearance has more negative impact on the well-being of the ocular surface (Afonso et al. 1999; Tsubota et al. 1999; Macri & Pflugfelder 2000; Dursun et al. 2002).

A reduction in clearance is often associated with symptoms of ocular irritation and can be due to organic or functional obstructions of the lacrimal passages, and non-obstructive factors such as punctual eversion and lacrimal pump insufficiency. Although irrigation and probing are widely used to evaluate the lacrimal drainage function and to establish the patency of the lacrimal drainage, they cannot provide information on milder obstructions or functional deficiencies (Sahlin & Chen 1996).

A number of tests have been developed to evaluate tear clearance and drainage. In the Jones test (Jones 1962), fluorescein solution is instilled into the tear film, and the tear clearance through the lacrimal passage is determined by measuring the dye recovery in the nasal cavity. However, in more than 20% of normal subjects, dye was not recovered thus limiting the value of this test (Zappia & Milder 1972). Fluorophotometry has been used to measure the decrease in the fluorescein concentration in the tear film over a 30-min period following instillation of 1 μl of 2% fluorescein sodium into the lower fornix of the eye. This method measures the tear turnover rate expressed as the

percentage decrease/min (van & Oosterhuis 1983; Webber et al. 1987; Pearce et al. 2001; Yen et al. 2001; Tomlinson & Khanal 2005). Because fluorophotometry requires a specific commercial instrument and the examination is time-consuming, simpler clinical tests have been designed, which use the same principle of dye disappearance. One representative method is the fluorescein clearance test developed by Xu and Tsubota. This test can provide the results of the Schirmer test and the tear clearance rate at the same time (Xu & Tsubota 1995). These dye clearance tests are either invasive or laborious, and the use of fluorescein has some limitation because the clearance of the tear fluids is not directly measured. Thus, a non-invasive, rapid, simpler tear clearance test that can be observed and measured is needed.

Applying a drop of lamp black suspension onto the eye, Maurice observed the existence of a steady flow in the lower tear meniscus towards the punctum. This flow, called Krehbiel flow, occurred after a blink when excess fluid is present in the conjunctival sac (Maurice 1973). Krehbiel flow is considered to be an effective drainage mechanism for reflex tearing. More importantly, its presence indicates a patency of the lacrimal drainage system. Therefore, we attempted to use this phenomenon to evaluate early phase tear clearance by monitoring the changes of tear meniscus parameters.

Fourier domain anterior segment optical coherence tomography (AS-OCT) is an imaging technique that provides cross-sectional views of the anterior segment of the eye with an acquisition time of 1.0–2.0 seconds and a resolution of about 10 μm (Steinert & Huang 2008). In addition, application of a swept source Fourier AS-OCT enhances the quality of the images by reducing the artefacts induced by eye movements. Sharp images of the tear meniscus can be observed and photographed rapidly and non-invasively. In addition, because swept source Fourier domain OCT use a 1310 nm wavelength light source, the scanning light is not visible to the patient, which could induce reflex tearing. Therefore, the dynamic changes of the tear can be monitored in real time and images photographed at any time selected while the subjects are being examined under natural blinking conditions.

In the literature, AS-OCT has been used to study the tear distribution in the eye, dynamic distribution of artificial tears (Wang et al. 2010), diurnal variations of the tear meniscus (Shen et al. 2008), age-related changes in the tear meniscus (Qiu et al. 2011) and the effect of airflow exposure on the tear meniscus (Koh et al. 2012). To date, there has been no report on the application of AS-OCT for tear clearance evaluations, and whether the values obtained are correlated with the clinical findings.

Thus, the purpose of this study was to determine whether the tear clearance rate can be evaluated by AS-OCT. To accomplish this, we dropped 5 μl of lukewarm saline solution into the lower conjunctival sac and recorded the changes of the tear meniscus by AS-OCT. To confirm that the tear clearance rates determined by AS-OCT were valid, we compared the tear clearance rate of young subjects to that of elder subjects, and also compared the rates to that obtained by more conventional methods. In addition, the relevance of some clinical findings assumed to be related to tear clearance was investigated.

Methods

Subjects

Sixty volunteers were divided into two groups: a young group that included 30 subjects (18 men and 12 women) with a mean age of 29.6 ± 7.2 years with a range of 20–59 years; and an elder group comprised of 30 subjects (17 men and 13 women) with a mean age of 71.4 ± 10.8 years with a range of 60–90 years. All participants were examined by slit-lamp biomicroscopy, and eyes with evident entropion or extropion, severe conjunctivochalasis (grade ≧ 3 as described below) and dry eye symptoms (determined by clinical examinations and questionnaire) were excluded. Also excluded were eyes with prior intra-ocular surgery, ocular trauma, ocular inflammation and abnormal intra-ocular pressure. Subjects with diabetic mellitus and those using any topical or systemic medications that could affect the tear secretion and drainage were excluded. Only the right eyes were examined and evaluated.

The procedures used conformed to the tenets of the Declaration of Helsinki. An informed consent was obtained from all subjects after an explanation of the nature and possible consequences of the procedures. The protocol used was approved by the Ethics Committee of Ehime University School of Medicine.

Anterior segment optical coherence tomography examinations

The examinations were carried out by an ophthalmologist who specializes in performing OCT examinations and was not part of the clinical examination team. Images were acquired by anterior segment swept source optical coherence tomography (SS-1000 CASIA). The acquisition time for all scans was 0.3 seconds with an axial resolution of approximately 8 μm and a transverse resolution of approximately 30 μm (Fukuda et al. 2013). The scan of the anterior segment was a non-contact procedure during which the subject fixated an internal target. The scan conditions were raster H scan range 16 mm, A/B scan 256 and B/C scan 256. The examination room was air-conditioned, and the temperature and humidity were kept as constant as possible.

The inferior tear meniscus was recorded with the vertical scan crossing the central cornea. Subjects were asked to blink naturally, and the tear meniscus was photographed between blinks for the baseline values. Then, 5 μl of lukewarm saline solution (0.9% sodium chloride solution, Otsuka Pharmacy, Osaka, Japan) was dropped into the lower conjunctival sac with a micropipette with care taken not to touch any ocular surface. An AS-OCT image was obtained immediately after the instillation (0_{second}), and subjects were then asked to blink naturally and were re-examined 30 seconds after the instillation. Each subject underwent three tests with an interval of at least 15 min between tests. The clearest image from each test was selected for the measurements and averaged. The results for all of the subjects were averaged for the statistical analyses.

Anterior segment optical coherence tomography image processing and data analyses

All images were processed and analysed by two graders (XZ, TK) who were masked to the demographics of the subjects. The average values of the two graders was used for the statistical analyses. The tear meniscus height

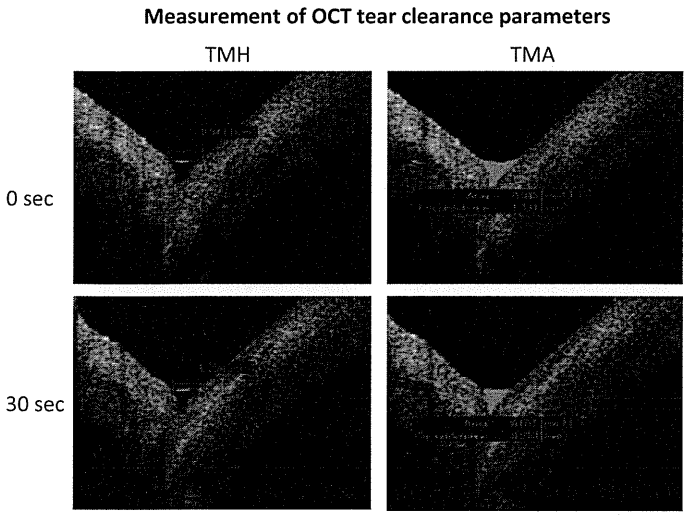

Fig. 1. Representative AS-OCT images showing how the early phase tear clearance was determined by measuring the tear meniscus height (TMH) and tear meniscus area (TMA). Values measured at 30 second following saline solution instillation (bottom panel) decreased compared with values obtained immediately after the instillation (0 second, top panel).

(TMH) and tear meniscus area (TMA) were measured using a built-in software program as described (Fukuda et al. 2013). Briefly, the TMH was defined as the distance between the upper and lower tear boundary line. The TMA was automatically calculated by the program after manually plotting the border of meniscus using at least 9 points (Fig. 1). These measurements had good reliability and reproducibility with the intra-observer and interobserver correlation coefficients ranging between 0.92 to 0.94 and 0.91 to 0.93, respectively. The AS-OCT tear clearance index was taken to be the percentage decrease in the TMH and TMA at 30 second following the application of the saline solution, that is, the TMH tear clearance rate (%) = ($TMH_{0\ second}$ − $TMH_{30\ second}$)/ $TMH_{0\ second}$ × 100%.

Correlations between OCT tear clearance and clinical parameters and fluorescein clearance test

Another group of 30 normal subjects (15 men and 15 women, mean age, 46.5 ± 14.2 years; range, 20–80 years) was studied. The inclusion and exclusion criteria were the same as that for the earlier subjects. All subjects were first evaluated with the AS-OCT tear clearance test, and then the following clinical features were determined. The grade of the conjunctivochalasis was determined according to the Yokoi classification as follows: grade 0, no conjunctivochalasis; grade 1, no conjunctivochalasis with natural blinks, chalasis aggravated after forced blink with chalasis height less than the height of tear meniscus; grade 2, chalasis apparent without forced blink with height less than the height of the tear meniscus; and grade 3, chalasis height higher than the tear meniscus height without forced blinking (Hirotani et al. 2003; Yokoi et al. 2003).

We also studied the correlations of OCT tear clearance with two new parameters that our pilot study had shown to be related to ageing and decreased tear clearance. (i) The protrusion of the inferior lacrimal punctum was graded as 1 for flat, 2 for mild protrusion, 3 for moderate protrusion and 4 for obvious protrusion. Representative slit-lamp biomicroscopic photographs are shown in Fig. 2A. (ii) The distance between the lacrimal punctum and the fluorescein stained line on the inner lid, the Marx line, was measured as follows: after staining the lower lid as described (Yamaguchi et al. 2006), the lid was pulled slightly downward and slit-lamp biomicroscopic photographs were taken with a ×16 objective lens and the photographs were digitally enlarged for measurements. The distance was measured using a calliper and corrected to the real value according to the magnification (Fig. 2B).

Finally, the fluorescein clearance test was also performed on the same office visit according to the method described in detail (Xu & Tsubota 1995). In brief, 10 μl of 0.5% fluorescein and 0.4% oxybuprocaine were dropped into the conjunctival sac, and the subjects were instructed to keep their eyes open for 5 min. A standard Schirmer strip was placed in the lower conjunctiva sac, and the eye was closed for another 5 min. The intensity of the staining of the strip was compared with a standard colour plate for a measure of the tear clearance rate. The tear clearance rate was determined by the rate at which the colour of the 0.5% fluorescein faded; graded as 1, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128 and lower. Data are presented in logarithmic values.

Statistical analyses

All data are expressed as the means ± standard deviations (SDs). Comparison of sex distribution differences between the young and elder groups was evaluated by the chi-squared test. Comparisons of other demographic data, TMH, TMA and tear clearance indices were evaluated by two-tailed Student's *t*-tests. A probability of $p < 0.05$ was considered statistically significant. Data were analysed with the JMP, version 10.0 for Windows statistical software (SAS Japan Inc., Tokyo, Japan).

Results

All of the AS-OCT images were clear, and the tear meniscus could be readily seen which made the measurements of the TMH and TMA easy with the built-in software. Our pilot studies had shown that the time required for the tear meniscus to return to the baseline after dropping 5 μl of saline solution into the conjunctival sac was about 2 min (Fig. 3). We also noted that the reduction in the TMH and TMA during the first 30 second was most significant. Therefore, the first 30 second was selected for the early phase tear clearance study.

There was no significant difference in the sex distribution between the young and elder groups ($p = 0.542$; chi-squared test). The baseline value of TMH was 0.21 ± 0.04 mm and that for TMA was 0.03 ± 0.01 mm^2. After 5 μl of saline was dropped into the conjunctival sac, the TMH measured immediately after instillation (0second) increased significantly to 0.41 ± 0.05 mm ($p = 0.003$) and the TMA to

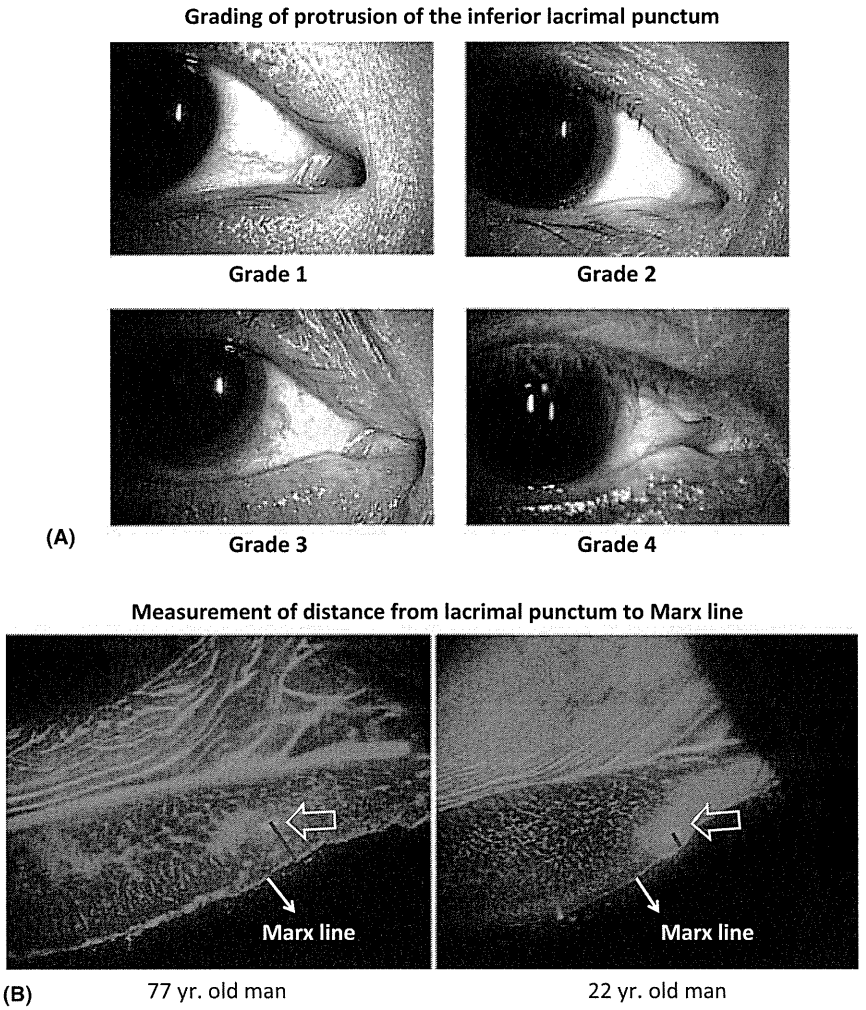

Fig. 2. Representative slit-lamp biomicroscopic photograph showing the grading method for protrusion of the inferior lacrimal punctum (A): Grade 1 = flat, 2 = mild protrusion, 3 = moderate protrusion and 4 = evident protrusion. Photograph showing how the distance (red lines) between the lacrimal punctum and the Marx line was measured (B) in an elder (left) and a young (right) subject. Open arrows indicate the inferior lacrimal punctum.

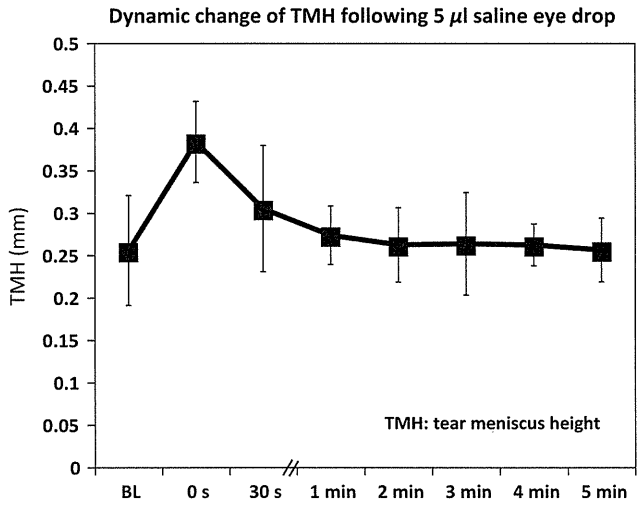

Fig. 3. Dynamics change of the tear meniscus height (TMH) following instillation of 5 μl of saline solution. BL: baseline.

0.07 ± 0.01 mm^2 ($p = 0.001$; Fig. 4). Following natural blinking for 30 seconds, both the TMH and TMA values decreased significantly compared with that at 0second ($p = 0.018$ and $p = 0.009$, respectively). The TMH tear clearance rate and TMA tear clearance rate were significantly correlated ($r = 0.820$, $p < 0.0001$; Spearman's correlation coefficient).

For the young group, the clearance rate for the TMH was 35.2 ± 11% and that for TMA was 28.1 ± 12.4%. In the elder group, the tear clearance rate for TMH was 12.4 ± 7.3% and that for TMA was 6.2 ± 9.1%. The differences in the TMH and TMA clearance rates between the two groups were significant ($p = 0.017$ and $p = 0.024$, respectively). When all subjects were analysed, age was significantly and negatively correlated with both the TMH clearance rate ($r = -0.522$, $p < 0.0001$; Fig. 5A) and the TMA clearance rate ($r = -0.417$, $p < 0.001$; Fig. 5B).

Both the TMH and TMA clearance rates were significantly and negatively correlated with the degree of conjunctivochalasis ($p = 0.004$ and $p = 0.012$, respectively), the distance between the inferior lacrimal punctum and Marx line ($p = 0.009$ and $p = 0.005$, respectively), and the degree of protrusion of the lacrimal punctum ($p = 0.019$ and $p = 0.027$, respectively). The correlation studies also showed a significant positive correlation between the OCT-determined clearance rates with the fluorescein clearance test ($r = 0.7019$; $p < 0.0001$ for TMH and $r = 0.3803$; $p = 0.0385$ for TMA; Fig. 6 and Table 1).

Discussion

An intact tear film is essential for the health and function of the eye, and normal tear film dynamics require adequate production of tears, retention of a tear film on the ocular surface and balanced elimination. Disruption of any of these components can lead to ocular surface disorders. For example, decreased production of tears accounts for the aqueous-deficient type of the dry eye syndrome and obstruction of the lacrimal drainage system accounts for epiphora. Therefore, a precise method for determining tear clearance is important for evaluating ocular surface disorders especially for dry eyes

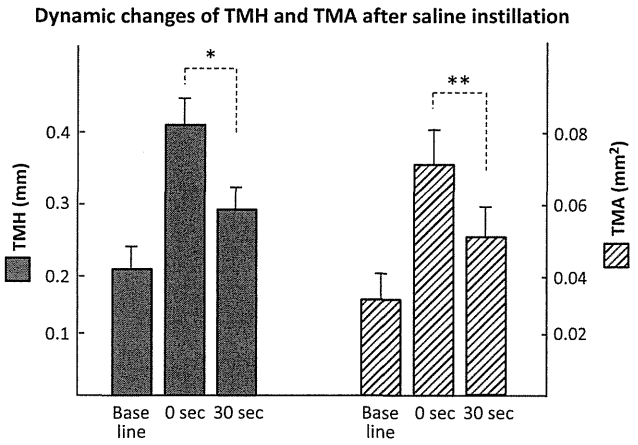

Fig. 4. Changes in the OCT-determined TMH (left vertical axis) and TMA (right vertical axis) tear clearance parameters following saline instillation. Sixty subjects (mean age 42.2 ± 11.3 years.) underwent AS-OCT examination. The tear meniscus was first recorded for the baseline value. Then, 5 μl of lukewarm saline solution was dropped into the conjunctival sac, the eye was re-examined immediately following the instillation (0second) and at 30 second following instillation (30 second). *p < 0.05, **p < 0.01.

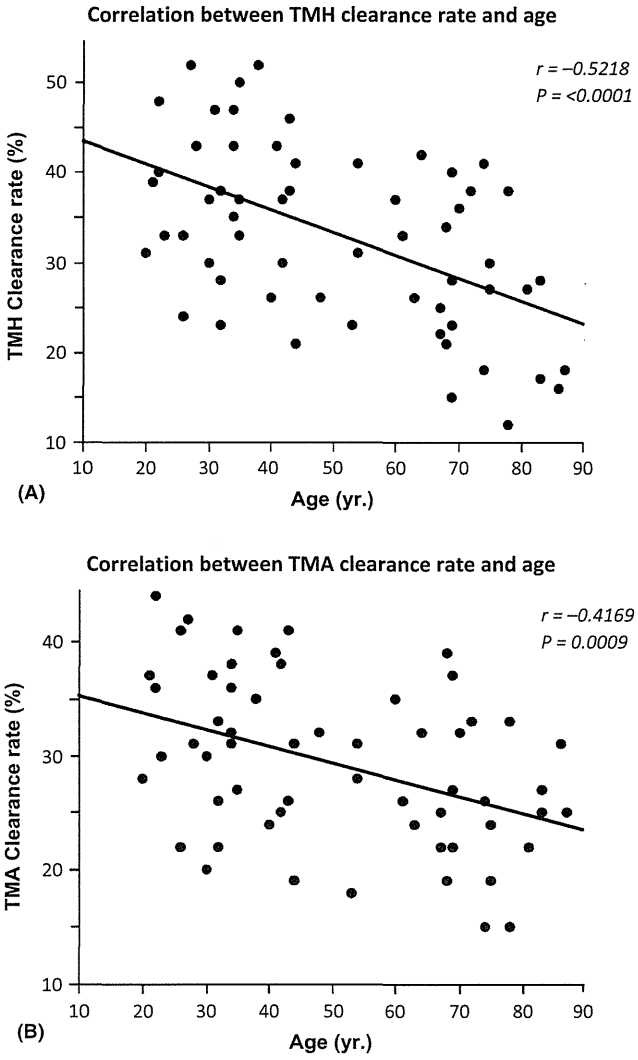

Fig. 5. Correlations between the OCT tear clearance rate of TMH (A) and TMA (B) and age. The OCT tear clearance rate was defined as the percentage decrease in the TMH or TMA at 30 second following saline instillation, for example tear clearance rate of TMH = ($TMH_{0second}$ − $TMH_{30second}$)/$TMH0_{second}$ × 100%. The Spearman's correlation coefficients and p values are shown.

and dysfunctions of the lacrimal drainage system.

Anterior segment optical coherence tomography can be used to obtain high-resolution cross-sectional views of the anterior segment of the eye, and the measurements of very small structures such as tear meniscus can be achieved in <1-second. In addition, AS-OCT has the advantage of being able to monitor eye movements, blinking and examining the status of the conjunctiva, for example conjunctivochalasis, in real time (Gumus et al. 2010).

The TMH and TMA are the parameters most frequently used to evaluate the morphology of the tear meniscus. They have been shown to be significantly reduced in patients with Sjögren syndrome (Qiu et al. 2012), and their values have been shown to be positively correlated with the Schirmer test results (Nguyen et al. 2012; Fukuda et al. 2013). Thus, these two parameters are the dimensions used to calculate the tear volume.

Our findings that TMH and TMA decreased most significantly at early phase following eye drop instillation suggested that our study most likely examined the effects of Krehbiel flow on the early phase tear drainage rather than the late-phase 'normal volume tear' clearance function.

The 30-second time was selected because our pilot study had shown that this was the minimal time for both TMH and TMA to be significantly decreased compared with the 0_{second} values. This was also a practical time for the examiner to obtain information such as the blink-related conjunctivochalasis and to photograph the tear meniscus. It was also short enough so that the tear volume would be less likely affected by other factors such as tear secretion and evaporation. In addition, this short test period should be less stressful for the patients.

Our findings showed that the TMH and TMA rates were significantly higher in younger than in the older subjects, which are in agreement with the results of fluorophotometry and fluorescein clearance tests (Mathers et al. 1996; Kaido et al. 2011). In addition, our data showed that the degree of conjunctivochalasis was significantly correlated with a decrease in the TMH and TMA tear clearance rates. This is reasonable as redundant conjunctiva tends to disturb the for-

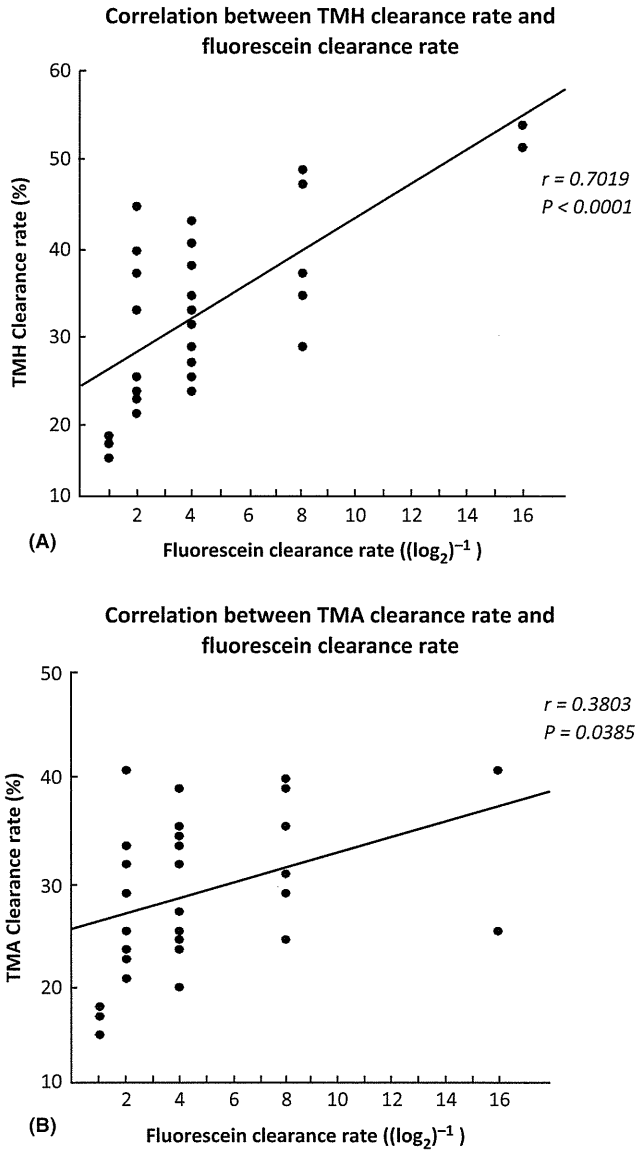

Fig. 6. Correlations between OCT tear clearance rate of TMH (A) and TMA (B) and fluorescein clearance rate. The fluorescein clearance rate is plotted in logarithmic values. The Spearman's correlation coefficients and p values are shown.

Table 1. Correlation of OCT tear clearance with clinical features.

	TMH		TMA	
	Spearman's correlation coefficient	p	Spearman's correlation coefficient	p
Degree of conjunctivochalasis	−0.6774	0.0035	−0.4537	0.0117
Distance of lacrimal punctum to marx line	−0.6295	0.0087	−0.6825	0.0046
Degree of protrusion of lacrimal punctum	−0.5831	0.0191	−0.4392	0.0273
Fluorescein clearance test	0.7019	<0.0001	0.3803	0.0385

mation of a tear meniscus as well as tear drainage into the lacrimal punctum. A similar conjunctivochalasis correlation was reported earlier by Wang et al. (2007).

Our study also showed that an increased distance between the inferior punctum and Marx line and protrusion of the inferior lacrimal punctum were significantly correlated with lower TMH and TMA tear clearance rates. We have reported that a forward shift of the Marx line represents an impaired meibomian gland function (Yamaguchi et al. 2006). However, this is not the case here because the tarsus is located away from the lacrimal punctum. Instead, the increased distance between the inferior punctum and Marx line may be related to the extent of undrained tear fluids during blinking. Using high-speed photography, Doane pointed out that the complete meeting of the upper and lower punctum during lid closure is important for efficient tear drainage into the lacrimal sac (Doane 1981). The protrusion of the lacrimal punctum may lead to an inadequate opposition of the upper and lower punctum, resulting in incomplete tear clearance. Further studies are needed to explore the relationship of these morphological changes with OCT tear clearance rate in the patients with dry eye or epiphora.

Our results showed that the OCT tear clearance tests gave quantitative values of the changes of the TMH and TMA. This new application of AS-OCT should increase its diagnostic abilities to evaluate the tear meniscus, tear clearance and drainage function. There are ongoing studies in our facility using this OCT-determined tear clearance method to evaluate patients with organic and functional occlusions of the lacrimal drainage system before and after surgical treatment.

There are limitations for this study. First, TMH and TMA are sectional scans of the tear meniscus, and measurements at a different location of the tear meniscus may be different. For increased reliability and to minimize measurement bias, we imaged all subjects at the central cornea and three tests were performed for each subject, and the average was used for the statistical analyses. These measurements had good reliability and reproducibility with high intra-observer and interobserver correlation coefficients.

Second, the tear meniscus changes observed may not all be caused by tear drainage. Although many factors such as absorption, secretion and evaporation can affect tear clearance, the only significant contribution should be drainage by the nasolacrimal system for our 30-second testing period.

Third, because the tears are not only distributed to the tear meniscus but

also to the pre-ocular tear film and the fornix, the tear meniscus could be affected by the tear flow from the fornix (Mishima et al. 1966; Fraunfelder 1976; MacDonald & Maurice 1991). Thus, the measurement of the changes in the tear meniscus should not be extrapolated to what happens to the entire tear volume. However, our data showed a significant correlation between the OCT tear clearance rate and fluorescein clearance test, indicating that it was an indirect evaluation of the tear clearance rate.

In summary, we have described a new application for AS-OCT that can evaluate the tear clearance in normal subjects. Our data showed this is a simple, rapid, non-invasive and quantitative method for evaluating early phase tear clearance. Further studies are needed to verify tear changes including pre-ocular tear film and the correlations of this OCT evaluation with other methods of tear clearance measurement in subjects with ocular surface disorders such as dry eye and lacrimal drainage occlusion.

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New side-view imaging technique for observing posterior chamber structures during cataract surgery in porcine eyes

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Abstract

Background: To develop a side-view imaging technique for observing the dynamic behavior of posterior chamber structures (PCSs) in porcine eyes which mimics closed-eye cataract surgery in humans.

Methods: Enucleated porcine eyes were placed into liquid nitrogen for 5 seconds and immediately bisected at about a 45-degree angle to the equatorial plane. The anterior portion was attached firmly to a glass slide with superglue and sprinkled with wheat flour. Phacoemulsification and aspiration (PEA) was performed as in humans on 10 consecutive porcine eyes. The movements of the PCSs were monitored through the glass slide with a high-resolution video camera set below the cut surface of the eye. The intraocular pressure (IOP) was monitored during the surgery. The highest IOP, operation time, and volume of irrigation fluid of 10 whole eyes were compared to that obtained from the bisected eyes glued to a glass slide. In a second set of experiments, the strength of the seal between the bisected eye and the glass slide was tested in three sets of eyes: 1) frozen eye fixed with superglue with wheat flour for 3 min; 2) frozen eye fixed with superglue for 3 min; and 3) non-frozen eye fixed with superglue for 30 min. The highest IOP that led to a disruption of the seal was compared among the three groups.

Results: PEA was successfully performed on 9 of 10 (90%) eyes with the movements of the PCSs clearly observed. The average maximum intraocular pressure of the 9 bisected eyes was 55.8 ± 4.7 mmHg and that for the 10 unbisected eyes was 55.3 ± 5.0 mmHg ($P = 0.650$). The frozen eye fixed with superglue in combination with wheat flour (Group 1) had the strongest sealing strength with an average IOP at the breaking point of 117.3 ± 36.2 mmHg.

Conclusions: Our side-view imaging technique can be used to evaluate the changes of the PCSs during intraocular surgery and for surgical training of new residents.

Keywords: Side-view imaging, Posterior chamber structures, Cataract surgery, Porcine eyes

Background

Over the past decade, phacoemulsification and aspiration (PEA) cataract surgery has become a safer and less invasive surgical procedure due to advances in surgical instruments and technology. To further minimize the intraoperative complications, it is necessary to understand how each surgical step might influence the intraocular structures especially the posterior chamber structures (PCSs), e.g., ciliary body, zonular fibers, anterior hyaloid membrane (AHM), and peripheral lens capsule. Because these structures are not visible to the surgeon during surgery, it is difficult to obtain information on their behavior during surgery. This is important because once these tissues are iatrogenically injured, a variety of complications can ensue. Therefore, new techniques to observe the behavior of the PCSs during surgery are necessary for better assessments of the surgery-related pathological changes.

The Miyake-Apple technique, originally developed in 1985 [1] and subsequently modified by Apple and

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Davis et al in 1990 [2], used postmortem human eyes and the preparation permitted real-time assessments of the intraoperative movements of the PCSs especially during the fixation of the intraocular lens. These methods have been widely used as research and educational tools to improve the quality and safety of PEA and cataract surgery.

However, the Miyake-Apple technique has some limitations. It enables the surgeon to observe only the lens and ciliary-zonular complex from the back of the eye, and this has limited the information on the behavior of the PCSs. In 1992, Assia and Apple developed an uveoscleral window technique in enucleated human eyes in which the lens and other PCSs could be seen by removing parts of the cornea, sclera, and ciliary body [3,4]. However, the behavior of PCSs could not be observed in closed-eye conditions by this method. In addition, a monitoring of the intraocular pressure (IOP) during the surgery was not possible.

To overcome these limitations, we have developed a new technique for examining the PCSs in postmortem porcine eyes. We were able to observe and video record the behavior of the PCSs continuously under closed-eye conditions as in human cataract surgery.

Methods

Preparation of porcine eyes

Porcine eyes were obtained from a local abattoir and were stored at 4°C until used. All eyes were used within 10 hours of enucleation. Each globe was carefully inspected and eyes with lacerations or perforations were excluded. Eyes were dipped into liquid nitrogen for 5 seconds while holding the cut end of the optic nerve with forceps. This made the outer surface of the globe firm and enabled us to bisect the globe without altering its shape. The globe was bisected with a sharp utility knife at a 45-degree to the equatorial plane (Figure 1A). The anterior portion was used. All experimental protocols were approved by the Ethical Committee of Ehime University and comply with the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research.

Then, approximately 0.2 grams of superglue (Aron alpha®, Toagosei, Japan) was applied in a circular pattern to a 50 mm × 50 mm pre-cleaned glass slide (Sharp cut filter® L37, HOYA Candeo Optronics, Japan). The glass slide was placed on the scleral rim taking care to avoid trapping air bubble (Figure 1B). After turning the slide over, 0.5 gram of wheat flour (Nissin Yuki®, Nissin Flour Milling Inc., Japan) was sprinkled onto the superglue as a bulking agent to promote solidification and to act as a filler of the interspaces. The eyes were left at room temperature for 3 minutes and a tight seal of the bisected eye to the glass slide developed.

Side-view imaging technique

The bisected eye glued to the glass slide was placed on a surgical table specially designed for this technique (Figure 1C and D). The table was inclined at 45° so that the corneal surface of the glued eye faced directly upward. A video camera with a zoom lens (Lens: AF Micro-Nikon 105 mm f/2.80, Nikon, Japan;

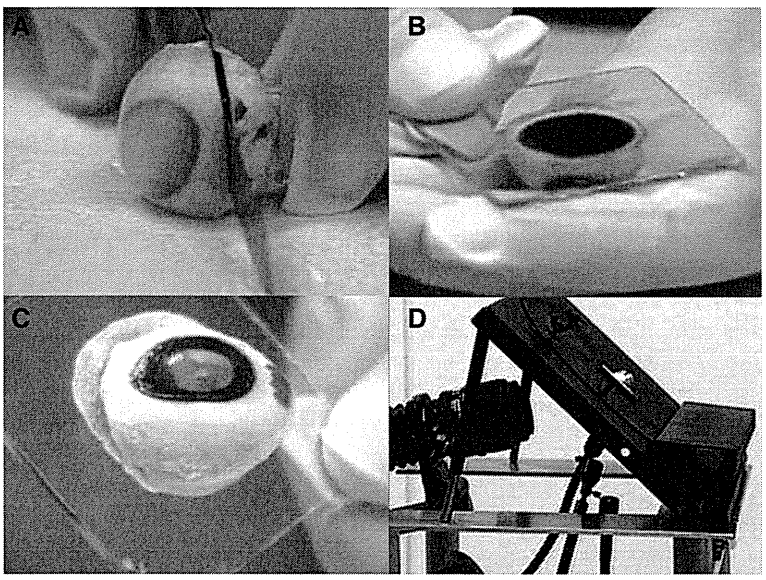


Figure 1 Preparation of an enucleated porcine eye and side-view imaging technique. A, The eye was frozen and transected at an angle of about 45 degrees to the equatorial plane with a utility knife. **B** and **C**, The bisected eye is fixed to a glass slide with a ring of superglue covered with wheat flour. The eye was left at room temperature for 3 minutes and a tight seal developed between the bisected eye and glass slide. **D**, Photograph of the equipment used for side-view cinematography.

Attachment: BELLOWS PB-6, Nikon, Japan; Camera: DSR-1, SONY, Japan) was set beneath the surgical table and the camera lens was focused on the PCSs. The posterior surface of the iris, posterior chamber, ciliary body, zonular fibers, and the equator of the lens were in focus. The surgeon's view camera was also connected and the images were video recorded simultaneously with the side-view images (BETACAM, SONY, Japan; Figure 1D).

Measurement of intraocular pressure (IOP)

The IOP of the operated eye was monitored continuously as described in detail [5]. In brief, a 2.4-mm corneal incision was made 1.5 mm central to the limbus at the 9-o'clock position. Then a 16-gauge needle attached to a pressure sensor (DI-151RS; DATAQ Instruments, Inc, Akron, Ohio, USA) was inserted into the anterior chamber (AC). Care was taken to avoid any leakage of the infusion fluid from the 16-gauge needle.

Phacoemulsification and aspiration procedure

After making a 2.8-mm corneal incision at the 12-o'clock position and a side port at the 2-o'clock position, the AC was filled with Healon® (AMO, USA). Then, a 5.5 to 6.0 mm continuous curvilinear capsulorrhexis was performed using capsulorrhexis forceps. The IOP was monitored through a 16-gauge needle attached to a pressure sensor in the AC. Hydrodissection was then performed (Nagahara cannula; ASICO Llc, Westmont, Illinois). The contents of the lens were emulsified with the bottle height at 75 cm, flow volume 26 ml/min, and aspiration rate 120 mmHg. The emulsified lens materials were removed by a Phacoemulsifier (Universal®, Alcon, USA) with a specially designed hook (MINAMI M-HOOK®, INAMI, Japan). Finally, the AC and the capsular bag were filled with Healon®, and an intraocular lens (AN6K®, Kowa Pharmaceutical, Japan) was implanted in the capsular bag using a lens forceps.

Evaluation of sealing strength of bisected porcine Eye glued to glass slide

For our technique, it was essential that the bisected eyes glued to the glass slide be able to withstand fluctuations in the IOP during the surgery. To determine which sealing method was the strongest, we assessed the three different methods of fixation. In Group 1, frozen eyes were bisected and fixed to the glass with superglue and wheat flour. In Group 2, the eyes were prepared in the same way except that wheat flour was not applied. In Group 3, non-frozen eyes were bisected and fixed to the glass slide with superglue alone and left undisturbed for about 30 minutes, the Miyake-view procedure. A sharp 27-gauge needle attached to a 5-ml disposable syringe and attached to a pressure sensor was inserted into the AC at the 12-o'clock position. Then balanced saline solution (BSS) was slowly injected into the AC to increase IOP until the eye-slide glass seal was broken. The maximal IOP at the breaking point was recorded. Ten eyes from each group were tested.

In another set of experiments, bisected eyes were compared to whole eyes. Ten eyes each were tested for the changes of IOP during each surgical step of PEA, and the highest IOP, operation time, and volume of irrigation fluid used were compared.

Statistical analyses

All data are expressed as the means ± standard deviations (SDs). Comparisons of the IOP among the groups tested for sealing strength of bisected eye glued to glass slide were evaluated by Tukey-Kramer's test. Comparisons of the IOP between bisected eye and whole eye group tested during PEA were evaluated by Mann-Whitney test. A probability level of $P < 0.05$ was considered statistically significant. Data were analyzed with the JMP version 8.0 for Windows statistical software (SAS Japan Inc., Tokyo, Japan).

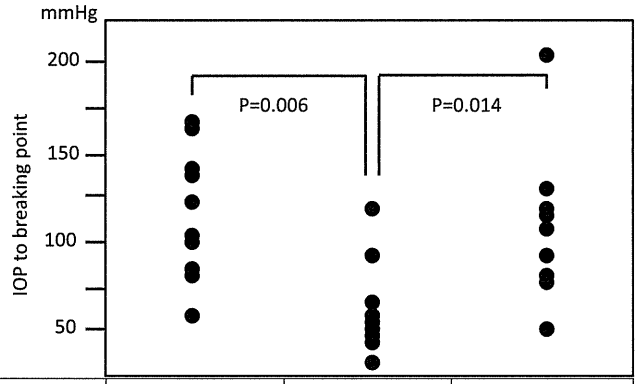
Results

Evaluation of sealing strength of bisected eye glued to glass slide

The average breaking pressure of the seal was 117.3 ± 36.2 mmHg in Group 1 (Figure 2), 64.1 ± 26.0 mmHg in Group 2, and 111.5 ± 40.5 mmHg in Group 3. The breaking pressure in Group 2 was significantly lower than that in Group 1 ($P = 0.006$) and Group 3 ($P = 0.014$, Tukey-Kramer's test). The breaking pressure in Group 1 was not significantly different from that of Group 3 ($P = 0.926$, Tukey-Kramer's test). Because the preparation of the bisected eyes with both superglue and wheat flour (Group 1) was rapid and effective, and because the eyes could tolerate the fluctuations in the IOP well, this method was used in all further experiments.

IOP changes during PEA

The IOP was monitored during PEA, and the changes in the IOP in representative cases of the whole eye and a bisected eye are shown in Figure 3. The fluctuations of the IOPs were similar for both groups. Although the shapes of graphs are somewhat different, the peaks of IOPs are comparable for all surgical procedure; viz, A, after setting the pressure sensor; B, during hydrodissection; C, during ultrasound sonication; D, during irrigation/aspiration; E, during IOL insertion; and F, during OVD aspiration (Figure 3). A summary of the highest IOP, operation time, and volume of irrigation solution used for the two groups is shown in Table 1. For bisected eyes, 9 of 10 (90%) eyes remained attached to the glass slide throughout the surgical procedures. The seal was broken in only one eye, and



	Group 1	Group 2	Group 3
frozen	+	+	−
Wheat flower	+	−	−
Time needed for sealing	3min.	3min.	30min.
Average IOP to breaking point(mmHg)	117.3±36.2	64.1±26.0	111.5±40.5

Tukey-Kramer's test

Figure 2 IOP values corresponding to loss of integrity of the eye-slide glass seal. The eye-glass slide seal was broken at a significantly lower IOP in frozen eyes with superglue and no wheat flour set for 3 minutes (Group 2) than that in Group 1 (P = 0.006) and Group 3 (P = 0.014, Tukey-Kramer's test). There was no significant difference in the IOP at the breaking point between Groups 1 and 3 (P = 0.926, Tukey-Kramer's test).

it occurred during ultrasound sonication of the lens. The average maximum IOP was 55.3 ± 5.0 mmHg for the whole eye group, and 55.8 ± 4.7 mmHg for the bisected eye group (P = 0.650, Mann-Whitney test). There was no significant difference in the average volume of the irrigation solution between the two groups (P = 0.107). The mean operation time was significantly longer in the bisected eyes than that for whole eyes (P = 0.028).

Observations of PCSs with side-view technique

The movements of the PCSs were clearly visible with our side-view technique. The movements of the surgical instruments during PEA were also clearly seen. For example, during the removal of the nucleus, we were able to observe the insertion of the MINMI M-hook® deep into the capsular bag which facilitated the removal of the lens content. The implantation of the intraocular

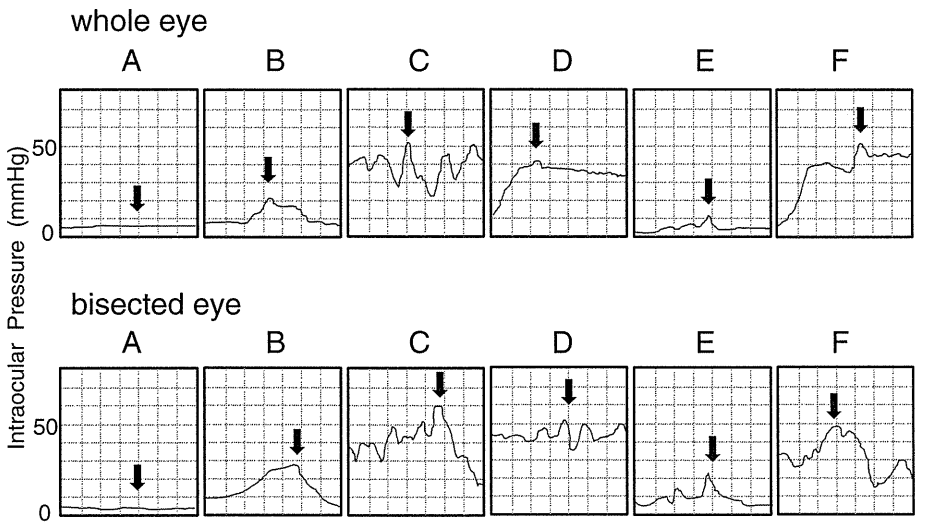


Figure 3 IOP fluctuations during surgery. A, After setting the pressure sensor, **B**, during hydrodissection; **C**, during ultrasound sonication (US); **D**, during irrigation/aspiration (I/A); **E**, during IOL insertion; **F**, during OVD aspiration. Top: Whole eye, A = 5.76 mmHg; B = 21.82 mmHg; C = 52.24 mmHg; D = 41.82 mmHg; E = 11.67 mmHg; and F = 52.01 mmHg. Bottom: Bisected eye; A = 4.85 mmHg; B = 28.03 mmHg; C = 59.70 mmHg; D = 52.27 mmHg; E = 23.33 mmHg; and F = 49.55 mmHg. Black arrows indicate peak IOPs. (X axis: 1 graduation = 0.5 seconds; Y axis: 1 graduation = 10 mmHg).

Table 1 Comparisons of parameters in whole and bisected eyes

Group	Highest IOP (mmHg)	Operation time (sec.)	Irrigation volume (ml)
Whole eye N=10	55.3±5.0	448.4±33.4	40.5±9.8
Bisected eye N=9	55.8±4.7	497.6±57.3	54.4±21.9
P value	0.650	0.028	0.107

IOP was monitored during PEA in two groups of porcine eyes (N=10 for each group). One group consisted of whole (unbisected) eyes, while the second group consisted of bisected eyes. The bisected eyes were glued to the glass slide with superglue and wheat flour. Among the bisected eyes, 9 of 10 (90%) eyes remained fixed to the slide throughout the procedure. The eye-glass slide seal was broken in only one case during ultrasound sonication of the lens. There was no significant difference between the two groups with respect to the maximum average IOP or the mean volume of the irrigation solution (Mann-Whitney test). The procedure time was significantly longer in bisected eyes than that of whole eyes ($P = 0.028$ Mann-Whitney test).

lens could also be seen in detail. Significant deformation of the zonular fibers indicated that the PCSs were under tension during these surgical maneuvers (Additional file 1).

Another advantage of this side-view imaging technique was that it allowed us to watch the flow of the irrigation solution through the bisected eye (Additional file 2). This was possible because the BSS irrigation fluid contained 1.0-μm fluorescein beads as described [5]. As shown in the first part of this video file, the fluorescein beads can be seen to be trapped in the zonular fibers (Additional file 2, Normal). This was probably due to the elevated IOP during the surgical procedures. During hydrodissection in some eyes, the space between the zonular fibers and anterior hyaloid membrane around the equatorial region of the lens increased, and numerous fluorescein beads were seen to scatter into the vitreous cavity through a tear in the anterior hyaloid membrane (AHM) around Wieger's ligament (Additional file 2, AHT).

Discussion

Damages to structures in the anterior chamber and central part of the posterior capsule can be clearly observed during intraocular surgery. However, it is difficult to see the damages in the posterior chamber, e.g., tearing of the zonular fibers [6,7]. Even if the stress on the zonules is transient, zonular weakness can lead to serious intra- or postoperative complications including zonular dialysis and vitreous prolapse. Although earlier reports have addressed the importance of the surgical tension on the zonular fibers and their surrounding tissues [8], the resilience and movements of the zonular fibers and AHM have not been well documented. One reason for this is the inability to view of the PCSs in a closed-eye surgical condition.

Our results showed that a side-view imaging system can provide useful information on the movements of the zonular fibers, the lens capsule, the ciliary body, and the AHM under a variety of surgical conditions during cataract surgery. Our technique is based on the elements of the Miyake-Apple posterior view and that of Assia and Apple side-view analysis of the lens [1–4]. However, our method has several advantages over these two methods.

First, cataract surgery can be successfully performed in a closed-eye surgery condition that mimics the surgical situation in human eyes. Khng et al. reported that the IOP was around 60-100 mmHg when the bottle height was at 130-150 cm during standard cataract phacoemulsification in cadaver eyes [9]. Our study showed that eyes prepared for side-view imaging system tolerated IOP elevations well and withstood pressure at a similar level of that of the whole eye for the tested period (Figure 3, Table 1). It is important that the side-view imaging technique with bisected eyes represent a practical surgical procedure in terms of having comparable values of the peak IOP as those of real "whole eye surgery". In addition, the surgery in the bisected eyes was satisfactory in terms of the operation time and volume of irrigation fluid solution used (Table 1). The average IOP at the breaking point (117.3 ± 36.2 mmHg) was much higher than the highest IOP measured during surgery for the bisected eyes and the whole eyes. At a 75 cm bottle height, there was no difference between the bisected eyes and the whole eyes.

Another advantage is that bisected eyes can be readily prepared by using our technique of superglue in combination of wheat flour. Our data showed that application of wheat flour was especially helpful in creating a secure sealing of the eye to the glass slide. The mechanism for this is not fully understood, however we suggest that the flour may act as a bulking agent to promote solidification and act as a filler of the interspaces. In addition, components in the wheat flour might facilitate or accelerate the polymerization of the cyanoacrylate in the glue. Therefore, an enhanced strength of sealing of the scleral rim onto the slide glass was achieved in a short time.

Our side-view imaging technique was used to monitor the behavior of the PCSs and movements of surgical instruments during cataract surgery. With this technique, surgeons should be able to perform PEA while simultaneously watching the movements of the surgical instruments such as the phaco-tip and nucleus chopper. Therefore, surgeons can easily confirm the proper depth of the surgical field as well as verify the correct maneuver of the surgical instruments. This side-view imaging technique should be able to be used for training residents on intraocular surgery or on improving surgical instruments or techniques for intraocular surgery.

We have reported on the benefits of using fluorescein beads in the irrigation solution during surgery [5]. The size of fluorescein beads is 1.0-μm in diameter, which is similar to that of bacteria. This method is particularly useful for tracing the flow of irrigation fluids during surgical procedures. We have demonstrated that as the