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Corneal protective effects of novel tear substitutes containing sodium hyaluronate and dodecahydrosqualene, squalane, in a porcine dry eye model

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ABSTRACT. To develop a novel tear substitute (TS) containing sodium hyaluronate (SH) and dodecahydrosqualene (DHS, squalane), we improved the prescription of a previously developed TS containing saline, 0.5% SH and 1% castor oil (CO), which had corneal protective effects against 60-min desiccation in a porcine dry eye model and viscosity of 106.8 mPa·S. Fresh porcine eyes were treated with a TS containing saline, 0.1%, 0.25%, 0.3% or 0.5% SH, and 1% CO or 1%, 2.5% or 5% DHS, and TS-treated eyes were desiccated for up to 180 min. The corneal damage was evaluated by the staining score of methylene blue (MB), absorbance of MB extracted from the cornea, the staining density of lissamine green (LG) and histopathology. The viscosities of the examined TS were also measured. A saline/0.5% SH/1% DHS solution had corneal protective effects for 90 min under desiccation and a viscosity of 110.0 mPa·s. A TS with saline, 0.1%, 0.25% or 0.3% SH and 1% or 2.5% DHS did not have better protective effects than a saline/0.5% SH/1% DHS solution, although a saline/0.3% SH/5% DHS solution exhibited greater corneal protection against 180-min desiccation on MB and LG staining and histopathological examination, and its viscosity was 34.5 mPa·s, which was similar to the 29.5 mPa·s of 0.3% SH. The saline/0.3% SH/5% DHS solution is available as a novel 3-hr long-lasting TS containing mucinomimetic and liquid oil components to treat and relieve dry eye symptoms in animals and humans.

KEY WORDS: corneal protective effects, dodecahydrosqualene (squalane), porcine dry eye model, sodium hyaluronate, tear substitutes

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The tear film consists of three components, a superficial lipid layer, middle aqueous layer and inner mucoid layer, and covers the surface of the cornea and conjunctiva, thereby maintaining wettability of the keratoconjunctiva, supplying nutrients and oxygen for the cornea, and providing an optically smooth surface for the cornea and antimicrobial activities for the ocular surface [3, 6, 8, 14, 27]. Deficiency of the tear film leads to keratokonjuntivitis sicca (KCS) in animals and dry eye syndrome (DES) in humans [3, 6, 14, 24, 27, 29]. In these pathological conditions, the diseased eyes usually undergo medical therapies, such as stimulation of natural tear production and/or replacement of the tear film, for which several tear substitutes (TSs) are available [3, 6, 14, 24]. The latter therapy using TSs usually requires medication more than 4–6 times per day or frequent administration, such as every 2 hr, in animal and human patients [6, 7, 14, 15]. However, TS therapy does not always achieve sufficient results due to the difficulty of frequent application [6, 14], suggesting that long-lasting TSs with fewer than 4–6 administrations per day are needed for effective and efficient treatment of KCS and DES in clinical practice, especially in the veterinary field. Therefore, we developed a novel TS containing saline, 0.5% sodium hyaluronate (SH) and 1% castor oil (CO) as a natural tear film mimetic solution with aqueous, mucoid and lipid components. However, this TS only provided 60 min of protection against desiccation of the cornea in a porcine dry eye model [10]. In addition, the saline/0.5% SH/1% CO solution was expected to not only be uncomfortable to use because of its high viscosity, but also to have a high material cost due to using 0.5% SH [10, 14]. The prescription of saline/0.5% SH/1% CO solution should be altered to enable comfort and reduce material cost, which means reducing the concentration of SH

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[10]. The oil product was also investigated to provide more effective corneal protection against desiccation than CO. We found that dodecahydrosqualene (DHS, squalane) has better corneal protective effects against desiccation than CO, suggesting DHS as a new alternative to CO [11]. Thus, we altered the prescription of TS containing saline, 0.5% SH and 1% CO solution to establish long-lasting TSs for improving the pathophysiological conditions of KCS or DES. The aim of this study was to create a novel TS containing saline, SH and DHS that has corneal protective effects against longer than 3- to 4-hr desiccation.

MATERIALS AND METHODS

Porcine eyes

Enucleated fresh porcine eyes with the surrounding eyelids and conjunctiva were purchased from the local abattoir in Osaka, Osaka Meat and Organs Co., Ltd. The eyes were placed in a cool box at 4°C with moist saline conditions (Otsuka Normal Saline, Otsuka, Tokyo, Japan) and transported to our laboratory at Osaka Prefecture University within an hour by motorcycle. Each porcine cornea was stained with 1% (W/V) fluorescein (FL) solution prepared with saline and sodium FL powder (Nacalai Tesque, Kyoto, Japan) to select eyes with an intact cornea. The selected eyes were gently rinsed with saline, the eyelids were closed and the eyes were stored in the moist cool box until the experiment.

Tear substitutes (TSs)

TSs were prepared with saline, CO (Sioe Pharmaceutical Co., Ltd., Hyogo, Japan), SH (Artz Dispo 25 mg, Seikagaku Corp., Tokyo, Japan) and DHS (squalane, FUJIFILM Wako Pure Chemical Corp., Osaka, Japan). TSs examined in this study were as follows: 1) saline, 2) saline/0.5% (V/V) SH/1% (V/V) CO or DHS solutions and 3) saline/0.1%, 0.25% or 0.3% (V/V) SH/1%, 2.5% or 5% (V/V) DHS solutions. To prepare respective TSs, individual reagents were simply mixed without additives. Each prepared TS was stored in a sterilized eye dropper, which was shaken well just before application to the porcine eyes.

Experimental procedures for the in vitro porcine dry eye model

The corneal protective effects of the TSs were evaluated using a modified procedure for a previously described porcine short-term dry eye model [10, 11]. The eyelids of the stored fresh porcine eyes were held open and excess saline was removed from the conjunctival sac with cotton swabs. Then, the eyes were securely positioned on a plastic cap with the corneal surface up and eyelids open in a chamber maintained at 20–22°C with 40–50% humidity without air flow in an experimental chamber, with environmental room conditions similar to those made with air-conditioners and dehumidifiers. The experimental eyes were treated with 2 drops (50 µl/drop) of an individual TS at the start of the experiment, blinked artificially a couple of times by closing and opening the eyelids, the eyelids were held open again and eyes were desiccated in the chamber for 60, 90 or 180 min. Using an automatic fluid delivery system for clinical practice, the control eyes as criteria were continuously treated with saline in the same chamber for the same maximal desiccation time (180 min). One experimental group with no treatment (NT) as the negative standard was desiccated for 180 min without the application of saline or TSs. The surrounding tissues of the eyes were removed after each desiccation period, and the corneas were stained by dipping in 1% (W/V) methylene blue (MB) solution prepared with sterilized distilled water (DW) and MB powder (Nacalai Tesque) or 1% (W/V) lissamine green (LG) solution made with sterilized DW and LG powder (Nacalai Tesque) for 1 min. MB and LG solutions were used to stain dead cells and cells with membrane damage in the cornea, respectively [5, 10, 11]. The stained eyes were washed well with saline, and the stained corneas were removed using surgical knives and scissors from the limbus of each eye. The MB-stained corneas were immediately photographed and then placed in 2 ml of acetone/saturated sodium sulfate solution (volume ratio of 7:3) for 16 hr at room temperature (22°C) to extract MB. The photographs were used to evaluate corneal staining scores (1 to 4) representing corneal integrity based on the extent of MB staining, as described previously [5, 10]. The absorbance of the extracted MB solutions was measured at 660 nm using a spectrophotometer (Smart Spec 3000, Bio-Rad, Hercules, CA, USA). The density of LG-stained corneas was measured using an image analyzer (FPD-100S, Fuji Film, Tokyo, Japan).

Histopathological examination

The harvested corneas for histopathological examination were fixed with 10% neutral buffered formalin, embedded in paraffin, sectioned at 3–4 µm, stained with hematoxylin and eosin (HE) after deparaffinization, and then examined microscopically.

Viscosity measurement

Viscosity of TSs was measured using an E-type viscometer with a cone-plate measuring device (TVE-20L, cone angle 1.34°, 24 mm diameter, Tokisangyo Co., Ltd., Tokyo, Japan). The sample viscosities were measured according to the instruction manual of the equipment. In brief, the viscometer was calibrated with the reference solution (standard liquid for calibrating viscometers JS 20, Nippon Grease Co., Ltd., Yokohama, Japan) for 2 min at a temperature of 20 ± 0.1°C and the number of revolutions of the rotor was 20 rpm. Then, 1.2 ml of each TS sample was placed in a sample cup of the viscometer, and its viscosity was measured under the same temperature of the reference solution for 1 min at different speeds of rotation of the cone-plate (0.5–100 rpm) using the up-down mode in duplicate or triplicate. The average values of duplicate or triplicate measurements were used as the measured viscosity values of examined TSs.

Statistical analysis

The corneal staining scores for MB, the absorbance of MB extracted from the cornea and the corneal staining density of LG are shown as the mean ± standard deviation (SD). Data of TS-treated eyes and no treatment eyes were compared with the control values of eyes continuously treated with saline, which were the model eyes with the conditions of live healthy animals. The data of examined eyes at the same desiccation time with different TSs were also compared statistically. Data were analyzed using one-way analysis of variance (ANOVA) for non-repeated measurements followed by Scheffe's test (Statcel 2nd ed.; OMS Publishing Co., Tokyo, Japan). A *P*-value less than 0.05 was considered to be significant.

In this experiment, the corneal protection time was defined as the maximum time without significant differences between the control eyes with no desiccation (CTS; continuous treatment with saline with 0 min of desiccation) and each experimental group under all MB and LG staining conditions.

RESULTS

The results of corneal protection by saline/0.5% SH/1% CO and saline/0.5% SH/1% DHS solutions in the porcine dry eye models are shown in Table 1. The staining score for MB, absorbance of MB extracted from the cornea and staining density of LG increased with longer desiccation times in eyes treated with both saline/0.5% SH/1% CO solution and saline/0.5% SH/1% DHS solution, although there were no significant differences in the MB staining score after 60-min desiccation, in absorbance of MB after 60- to 180-min desiccation, or in LG staining density after 60- or 90-min desiccation between CTS and saline/0.5% SH/1% CO solution-treated eyes, suggesting that a saline/0.5% SH/1% CO solution had 60-min protective effects against corneal desiccation, as previously reported [10]. On the other hand, there were no significant differences in all MB and LG staining parameters up to 90-min desiccation between the control eyes with no desiccation and saline/0.5% SH/1% DHS solution-treated eyes, demonstrating that the saline/0.5% SH/1% DHS solution had 90-min corneal protective effects on the porcine dry eye model.

Next, altered prescriptions of TSs containing DHS were examined to establish long-lasting TSs with a SH concentration of less than 0.5%. The corneal protective effects by TSs containing saline, 0.1%, 0.25% or 0.3% SH and 1% DHS in the dry eye models are shown in Table 2. There were significant differences in the MB staining score after 90- and 180-min desiccation, in absorbance of MB extracted from the cornea after 180-min desiccation and in LG staining density after 60- to 180-min desiccation between the control eyes and saline/0.1% or 0.25% SH/1% DHS solution-treated eyes. The saline/0.3% SH/1%DHS solution significantly increased values of MB staining parameters after 180-min desiccation, and LG staining parameters after 90- and 180-min desiccation. However, the duration of its corneal protection was only 60 min, which was 30 min shorter than that of a saline/0.5% SH/1% DHS solution.

Saline/0.1% or 0.25% SH/2.5% DHS solutions provided 60-min corneal protection based on MB staining parameters, similar to saline/0.1% or 0.25% SH/1% DHS solutions, and their corneal protective effects were confirmed by the LG staining density after

Table 1. Corneal protective effects of tear substitutes containing saline, 0.5% sodium hyaluronate and 1% castor oil or 1% dodecahydrosqualene (squalane)

Desiccation times (min)	Control (each sample number: 14–19)	Saline/0.5% SH/1% CO (each sample number: 8)	Saline/0.5% SH/1% DHS (each sample number: 8)
Methylene blue corneal staining score			
0 (CTS)	1.1 ± 0.2	-	-
60	-	1.0 ± 0	1.0 ± 0
90	-	1.9 ± 0.6*	1.4 ± 0.5
180	-	2.3 ± 0.5*	2.0 ± 0.5*
NT (180-min desiccation)	4.0 ± 0*	-	-
Absorbance of methylene blue extracted from the cornea at 660 nm			
0 (CTS)	0.043 ± 0.014	-	-
60	-	0.063 ± 0.007	0.055 ± 0.011
90	-	0.088 ± 0.016	0.071 ± 0.008
180	-	0.105 ± 0.016	0.094 ± 0.022
NT (180-min desiccation)	0.247 ± 0.082*	-	-
Lissamine green corneal staining density			
0 (CTS)	0.229 ± 0.006	-	-
60	-	0.258 ± 0.007	0.241 ± 0.008
90	-	0.268 ± 0.010	0.251 ± 0.006
180	-	0.295 ± 0.016*	0.276 ± 0.009*
NT (180-min desiccation)	0.451 ± 0.027*	-	-

SH: sodium hyaluronate, CO: castor oil, DHS: dodecahydrosqualene (squalane), CTS: continuous treatment with saline (0 min of desiccation), NT: no treatment with 180-min desiccation. An asterisk indicates significant differences ($P<0.05$) between CTS and each experimental group.

Table 2. Corneal protective effects of tear substitutes containing saline, sodium hyaluronate (0.1–0.3%) and 1% dodecahydrosqualene (squalane)

Desiccation times (min)	Control (each sample number: 14–19)	Saline/0.1% SH/1% DHS (each sample number: 8)	Saline/0.25% SH/1% DHS (each sample number: 8)	Saline/0.3% SH/1% DHS (each sample number: 8)
Methylene blue corneal staining score				
0 (CTS)	1.1 ± 0.2	-	-	-
60	-	1.5 ± 0.5	1.7 ± 0.5	1.5 ± 0.5
90	-	2.5 ± 0.5*	2.2 ± 0.8*	1.9 ± 0.4
180	-	3.4 ± 0.5*	3.0 ± 0.5 *	2.5 ± 0.5*
NT (180-min desiccation)	4.0 ± 0*	-	-	-
Absorbance of methylene blue extracted from the cornea at 660 nm				
0 (CTS)	0.043 ± 0.014	-	-	-
60	-	0.075 ± 0.016	0.078 ± 0.022	0.078 ± 0.013
90	-	0.099 ± 0.012	0.103 ± 0.014	0.100 ± 0.008
180	-	0.156 ± 0.027*	0.150 ± 0.015*	0.149 ± 0.021*
NT (180-min desiccation)	0.247 ± 0.082*	-	-	-
Lissamine green corneal staining density				
0 (CTS)	0.229 ± 0.006	-	-	-
60	-	0.261 ± 0.011*	0.263 ± 0.007*	0.244 ± 0.022
90	-	0.280 ± 0.009*	0.266 ± 0.009*	0.279 ± 0.014*
180	-	0.304 ± 0.013*	0.290 ± 0.009*	0.295 ± 0.005*
NT (180-min desiccation)	0.451 ± 0.027*	-	-	-

SH: sodium hyaluronate, DHS: dodecahydrosqualene (squalane), CTS: continuous treatment with saline (0 min of desiccation), NT: no treatment with 180-min desiccation. An asterisk indicates significant differences ($P<0.05$) between CTS and each experimental group.

Table 3. Corneal protective effects of tear substitutes containing saline, sodium hyaluronate (0.1–0.3%) and 2.5% dodecahydrosqualene (squalane)

Desiccation times (min)	Control (each sample number: 14–19)	Saline/0.1% SH/2.5% DHS (each sample number: 8)	Saline/0.25% SH/2.5% DHS (each sample number: 8)	Saline/0.3% SH/2.5% DHS (each sample number: 8)
Methylene blue corneal staining score				
0 (CTS)	1.1 ± 0.2	-	-	-
60	-	1.4 ± 0.5	1.4 ± 0.5	1.3 ± 0.5
90	-	2.1 ± 0.6*	1.9 ± 0.6*	1.6 ± 0.5
180	-	2.6 ± 0.5*	2.5 ± 0.5*	2.3 ± 0.5*
NT (180-min desiccation)	4.0 ± 0*	-	-	-
Absorbance of methylene blue extracted from the cornea at 660 nm				
0 (CTS)	0.043 ± 0.014	-	-	-
60	-	0.083 ± 0.013	0.076 ± 0.009	0.079 ± 0.008
90	-	0.103 ± 0.007	0.105 ± 0.021	0.113 ± 0.014
180	-	0.140 ± 0.015*	0.151 ± 0.013*	0.125 ± 0.018*
NT (180-min desiccation)	0.247 ± 0.082*	-	-	-
Lissamine green corneal staining density				
0 (CTS)	0.229 ± 0.006	-	-	-
60	-	0.250 ± 0.013	0.250 ± 0.008	0.243 ± 0.007
90	-	0.264 ± 0.012*	0.263 ± 0.009*	0.261 ± 0.006*
180	-	0.298 ± 0.015*	0.286 ± 0.007*	0.280 ± 0.008*
NT (180-min desiccation)	0.451 ± 0.027*	-	-	-

SH: sodium hyaluronate, DHS: dodecahydrosqualene (squalane), CTS: continuous treatment with saline (0 min of desiccation), NT: no treatment with 180-min desiccation. An asterisk indicates significant differences ($P<0.05$) between CTS and each experimental group.

60-min desiccation (Table 3). There were no significant differences in MB staining parameters after 60- or 90-min desiccation, or in LG staining parameters after 60-min desiccation between the control eyes without desiccation and saline/0.3% SH/2.5% DHS solution-treated eyes (Table 3). This suggested that modified prescriptions of saline/0.1%, 0.25% or 0.3%SH/2.5% DHS have only 60-min corneal protective effects against desiccation and they are not more useful.

The corneal protective effects of saline/0.1%, 0.25% or 0.3% SH/5% DHS solutions in the porcine dry eye model are shown in Table 4. A saline/0.1% SH/5% DHS solution had corneal protective effects for up to 90 min based on two MB parameters and up to 60 min based on LG staining, indicating that the duration of its corneal protection was 60 min. There were no significant differences

Table 4. Corneal protective effects of tear substitutes containing saline, sodium hyaluronate (0.1–0.3%) and 5% dodecahydrosqualene (squalane)

Desiccation times (min)	Control (each sample number: 14–19)	Saline/0.1% SH/5% DHS (each sample number: 8)	Saline/0.25% SH/5% DHS (each sample number: 8)	Saline/0.3% SH/5% DHS (each sample number: 8)
Methylene blue corneal staining score				
0 (CTS)	1.1 ± 0.2	-	-	-
60	-	1.3 ± 0.5	1.2 ± 0.4	1.0 ± 0
90	-	1.8 ± 0.5	1.3 ± 0.5	1.1 ± 0.4
180	-	2.4 ± 0.5*	2.3 ± 0.5*	1.5 ± 0.5‡
NT (180-min desiccation)	4.0 ± 0*	-	-	-
Absorbance of methylene blue extracted from the cornea at 660 nm				
0 (CTS)	0.043 ± 0.014	-	-	-
60	-	0.075 ± 0.005	0.061 ± 0.010	0.048 ± 0.017
90	-	0.094 ± 0.009	0.091 ± 0.006	0.090 ± 0.011
180	-	0.118 ± 0.010*	0.108 ± 0.014	0.100 ± 0.012
NT (180-min desiccation)	0.247 ± 0.082*	-	-	-
Lissamine green corneal staining density				
0 (CTS)	0.229 ± 0.006	-	-	-
60	-	0.235 ± 0.012	0.240 ± 0.011	0.215 ± 0.009
90	-	0.261 ± 0.008*	0.259 ± 0.006*	0.243 ± 0.009
180	-	0.288 ± 0.013*	0.280 ± 0.014*	0.259 ± 0.010
NT (180-min desiccation)	0.451 ± 0.027*	-	-	-

SH: sodium hyaluronate, DHS: dodecahydrosqualene (squalane), CTS: continuous treatment with saline (0 min of desiccation), NT: no treatment with 180-min desiccation. An asterisk indicates significant differences ($P<0.05$) between CTS and each experimental group. A double dagger indicates significant differences between saline/0.1% or 0.25% SH/5% DHS groups and saline/0.3% SH/5% DHS group ($P<0.05$).

in the MB staining score after up to 90-min desiccation, in MB absorbance after up to 180-min desiccation or in LG staining density after only 60-min desiccation between eyes without desiccation and saline/0.25% SH/5% DHS solution-treated eyes, suggesting that saline/0.25% SH/5% DHS solution had 60-min corneal protective effects against desiccation. No significant differences were confirmed in all MB and LG staining parameters after up to 180-min desiccation between control eyes with CTS and saline/0.3% SH/5% DHS solution-treated eyes. The MB staining score of the saline/0.3% SH/5% DHS solution-treated group was also significantly lower than those of the saline/0.1% or 0.25% SH/5% DHS solution-treated groups. This suggested that saline/0.3% SH/5% DHS solution not only has 180-min corneal protective effects against desiccation, but that it is also a good candidate for a long-lasting TS to treat eyes with tear film abnormalities and/or dry eye conditions.

The viscosity of the components of TSs and major TSs used in this experiment was examined, and the results are shown in Table 5. The viscosity of 0.1% and 0.3% SH solutions was 6.4 mPa·S and 29.5 mPa·S, respectively. The viscosity of 0.5% SH was 109.2 mPa·S, and 1% or 5% CO and 1% or 5% DHS solutions had a similar viscosity to saline, as shown in Table 5. The viscosities of saline/0.5% SH/1% CO, saline/0.5% SH/1% DHS and saline/0.3% SH/5% DHS solutions were 106.8 mPa·S, 110.0 mPa·S and 34.5 mPa·S, respectively, and that of a saline/0.3% SH/5% DHS solution was similar to that of 0.3% SH. A saline/0.1% SH/5% DHS solution had a viscosity of 5.1 mPa·S, which was similar to that of 0.1% SH solution (6.4 mPa·S).

Table 5. Viscosity of the components of tear substitutes and major tear substitutes examined

Tear substitutes	Viscosity (mPa·S)
Saline (S)	1.1
Sodium hyaluronate (SH)	
0.1%	6.4
0.3%	29.5
0.5%	109.2
Castor oil (CO)	
1%	1.1
5%	1.2
Dodecahydrosqualene (DHS)	
1%	1.1
5%	1.4
Combined tear substitutes	
S/0.5% SH/1% CO	106.8
S/0.5% SH/1% DHS	110.0
S/0.3% SH/5% DHS	34.5
S/0.1% SH/5% DHS	5.1

Histopathological examinations revealed the corneal protective effects of saline/0.3% SH/5% DHS solution in the porcine dry eye model (Fig. 1). There were no histopathological changes in corneal epithelial cells of the non-experimental porcine eyes (Fig. 1A). Minimal histopathological alterations with mild cytoplasmic vacuolations were detected on the basal and wing cells in the corneal epithelial layer in the eyes continuously treated with saline (Fig. 1B). On the other hand, the eyes without treatment (no treatment eyes) after 180-min desiccation exhibited significant histopathological alterations consisting of marked swelling of basal and wing cells, which had both cytoplasmic vacuolation and nuclear swelling when compared with the control corneas continuously administered saline (Fig. 1C). The condition of the corneal epithelium in the porcine eyes treated with a saline/0.5% SH/1% CO solution after 180-min desiccation was similar to that of eyes desiccated for the same time, although the degree of alteration in the saline/0.5% SH/1% CO solution-treated group was milder than that in the groups without TS treatment, including saline (Fig. 1D). Saline/0.5% SH/1% DHS solution-treated eyes had fewer histopathological changes than those administered

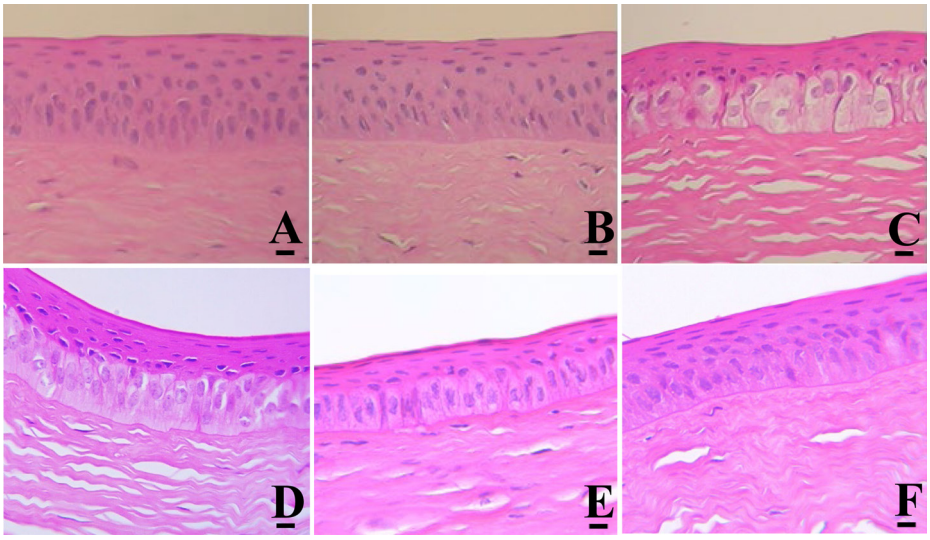

Fig. 1. Typical histopathology of the cornea administered major tear substitutes containing saline, sodium hyaluronate and dodecahydrosqualene after 180-min desiccation using the porcine dry eye model [A; immediately after harvest, B; continuous treatment with saline for 180 min, C; no saline or tear substitute treatment, D; treatment with saline/0.5% sodium hyaluronate (SH)/1% castor oil (CO), E; treatment with saline/0.5% SH/1% dodecahydrosqualene (DHS, squalane), F; treatment with saline/0.3% SH/5% DHS]. There are no histopathological abnormalities in the cornea of the eye immediately after harvest (**A**). Minimal histopathological alterations are detected on the basal and wing cells in the corneal epithelial layer in the eye continuously treated with saline (**B**), whereas the untreated eye (no treatment eye) has significant histopathological alterations consisting of marked swelling of basal and wing cells, with cytoplasmic vacuolation and nuclear swelling (**C**). The abnormalities of the corneal epithelium in the examined eye treated with saline/0.5% SH/1% CO solution are milder than those in the no treatment eye (**D**). The saline/0.5% SH/1% DHS solution-treated eye has milder histopathological changes than the eye administered saline/0.5% SH/1% CO solution (**E**). There are minimal histopathological abnormalities in the corneal basal and wing cells, with mild cytoplasmic vacuolation compared with the eye with continuous saline treatment (**F**). Bar=10 μm.

a saline/0.5% SH/1% CO solution (Fig. 1E). Marked corneal protection was noted with saline/0.3% SH/5% DHS solution in porcine eyes, which exhibited mild cytoplasmic vacuolation of basal and wing cells on the corneal epithelium compared with eyes continuously treated with saline (Fig. 1F).

DISCUSSION

There are many commercially available TSs containing one or more tear components, such as aqueous, mucin and/or lipid components [6, 7, 24], and new lacrimomimetics are emerging on the market in both veterinary and medical fields. A large number of basic and clinical research studies have been performed to demonstrate the efficacies of TSs for treating KCS or DES [7, 21]. To our knowledge, most previous studies of TSs only indicated the frequency of administration of the examined TSs and did not describe the exact duration of the corneal tissue protection [6, 7, 21]. Frequent administration of TSs more than 4–6 times per day is still recommended to achieve a good therapeutic outcome for KCS or DES [6, 7, 14, 15]. However, such frequent application should be reduced to improve patient compliance in clinical practice, enabling reliable administration of TSs and increasing the efficiency of tear replacement therapy for animals with KCS or DES patients. To estimate the appropriate administration interval of TSs, the exact duration of corneal tissue protection was evaluated using an *in vitro* porcine short-term dry eye model, although TSs with long corneal protective effects were not developed in the previous study [10, 11].

The prescription of the TS mimicking natural tear film with aqueous, mucin and lipid components was reconsidered to make a novel TS with effects lasting more than 3 to 4 hr. It is well known that SH is a mucinomimetic product, which is one of the main ingredients of TSs, and it provides good protective effects via its excellent viscoelastic and lubricating properties on the desiccated keratoconjunctiva of eyes with KCS or DES [6, 7, 23]. The concentrations of SH preparations are mainly 0.1–0.4% in current clinical practice [6, 7, 14, 28], and 0.3% SH solution exhibited better corneal protective effects than 0.1% and 0.18% SH preparations in a murine dry eye model [30]. In addition, 0.4% SH artificial tears are recommended to be used in the treatment of KCS animals [14]. On the other hand, one study demonstrated that the time of corneal protection was only 60 min for 1% SH and less than 60 min for 0.5% SH in the porcine dry eye model [10], suggesting that a higher concentration of SH is not always effective for corneal protection against desiccation. A higher concentration of SH will not only increase the price of TS products, but also increase their viscosity [14, 23]. The higher viscosity of SH may induce blurred vision due to non-uniform distribution of SH on the corneal surface of the eye [23]. The clinically available and applicable SH concentration may be 0.3–0.4%, which is used in commercial products [7, 14, 28, 30]. Therefore, SH at 0.3% or less was used in novel TS prescriptions established in this study.

As water retention of SH depends on its concentration, lower concentrations of SH, such as 0.3%, have a lower water retention capacity than 0.5% or 1% SH [9, 23]. We accounted for the decreased water retention of 0.3% or less SH by covering the surface with an oil product to ensure water retention capacity. CO was initially used as the oil product in the TS mimicking natural tear film, although the TS with SH and CO did not provide long-lasting corneal protection [10]. On the other hand, DHS was more effective against desiccation and 5% DHS exhibited 60-min protective effects against corneal desiccation, similar to solutions of saline/0.5% SH/1% CO and/or 1% SH in our previous studies [10, 11]. This performance was considered to be due to the characteristics of DHS as an oil product with good water retention capacity [13]. DHS may help prevent corneal drying because it is an oil product that is more lubricious and spreads wider than CO on the water surface [2, 4]. Moreover, DHS can penetrate the lipid bilayer of the cell membranes because of its higher cell permeability and because it is one of the constituents of cell membranes, thereby providing cell-protective and membrane stabilizing effects to the corneal epithelium [12]. The reduced histopathological changes, such as mild cytoplasmic vacuolation of basal and wing cells, may be associated with cell-protective and membrane stability effects of DHS, and water retention capacity of DHS and SH [7, 12, 30].

The saline/0.3% SH/5% DHS solution had good and long protective effects on the cornea against desiccation for 180 min in the porcine dry eye model (Table 4 and Fig. 1), and such long-lasting corneal protection was the goal of this study. The properties of the novel modified TS may be attributed to the additive effects of SH with viscoelastic and lubricating properties, and DHS having lubricating and cell-protective effects associated with cell-membrane stabilization [6, 7, 12, 21, 23, 28]. This extended 3-hr protection was marked when compared with the saline/0.5% SH/1% CO solution having 60-min protection against the same corneal desiccation [10]. Complete dry eye conditions were observed in eyes without tear films and without blinking in the presented study. Therefore, the effective time of the saline/0.3% SH/5% DHS solution is expected to be longer in clinical practice because there may be few clinical cases of complete dry eye conditions in the veterinary and medical fields. The viscosity of the mixture with saline, 0.3% SH and 5% DHS was 34.5 mPa·s, which was similar to that of 0.3% SH with 29.5 mPa·s (Table 5), and it may be acceptable for clinical use because there is a commercial product containing 0.3% SH solution in Japan. The saline/0.3% SH/5% DHS solution is one candidate for a long-lasting TS for the treatment of KCS and DES in future clinical practice.

The solutions of saline/0.3% SH/1% DHS, saline/0.1–0.3% SH/2.5% DHS and saline/0.1% or 0.25% SH/5% DHS provided short-term protective effects on the cornea against desiccation for 60 min, similar to saline/0.5% SH/1% CO solution and 5% DHS in the dry eye model (Tables 2–4) [11]. These corneal protective effects were poorer than those of a saline/0.3% SH/5% DHS solution, although the prescriptions with a low concentration of SH and/or DHS, especially saline/0.1% SH/5% DHS solution, may be used in KCS/DES cases with mild dry eye conditions in clinical practice. In addition, a low-concentration formulation of saline/0.1% SH/5% DHS had a lower viscosity of 5.1 mPa·S, which was similar to the 6.4 mPa·S of commercially available 0.1% SH (Table 5). The formulation with a low viscosity will be useful to prevent blurred vision in animals/patients using a TS containing SH and DHS [23]. SH and DHS-containing TSs can be applied to a wide range of KCS or DES cases if there are different products containing high or low concentrations of SH as a new product line.

Our study has some limitations associated with enucleated porcine eyes, which have no biological reactions. Blinking is an essential function to maintain tear film on the cornea, thereby maintaining a uniform thickness of the tear film and tear dynamics, including its volume [16, 17, 25]. The distribution and/or dynamics of the instilled TSs may not be accurately evaluated in the enucleated porcine eyes without blinking ability. In addition, we were unable to investigate important inflammatory reactions related to the pathophysiology of KCS or DES. Numerous cytokines and chemokines are induced via corneal inflammation associated with increased osmolality of the tear film under dry eye conditions [1, 18, 19, 22, 31]. These inflammatory reactants can be suppressed by reducing tear osmolality with tear replacement therapy using TSs [20, 26]. However, it is impossible to demonstrate the association of TSs with the reduction of corneal inflammation in the dry eye model we used. Therefore, the corneal protective effects of the novel TSs containing 0.3% SH and 5% DHS should be evaluated multilaterally in an *in vivo* experimental model using live animals or in a clinical trial in the future.

In conclusion, the saline/0.3% SH/5% DHS solution, a newly modified novel TS prescription containing SH and DHS, had long protective effects on the cornea against desiccation for 180 min in a porcine dry eye model, suggesting that it is applicable as a novel TS mimicking natural tear film with aqueous, muchinomimetic product and liquid oil components to treat and relieve pathological conditions of KCS in animals or human DES.

CONFLICT OF INTEREST. The authors have no conflicts of interest to disclose.

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