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Corneal-Protective Effects of an Artificial Tear Containing Sodium Hyaluronate and Castor Oil on a Porcine Short-Term Dry Eye Model

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ABSTRACT. The corneal-protective effects of an artificial tear containing sodium hyaluronate (SH) and castor oil (CO) were evaluated on a porcine short-term dry eye model. Fresh porcine eyes with an intact cornea were treated with an artificial tear of saline, SH solution (0.1%, 0.5% or 1%), CO solution (0.5%, 1% or 5%) or a mixture solution containing 0.5% SH and 1% CO and then desiccated for 60, 90 or 180 min. To assess corneal damage, the eyes were stained with methylene blue (MB) or lissamine green (LG). The staining score of MB, absorbance of MB extracted from the cornea and staining density of LG increased significantly with increasing desiccation time in untreated and all artificial tear-treated eyes, although there were no significant differences in staining scores and absorbance of MB between eyes treated continuously with saline and 1% SH-treated ones at 60 and 90 min of desiccation or the mixture-treated eyes at 60 min of desiccation. No significant differences in the staining density of LG were also found between continuous saline-treated eyes and ones desiccated for 60 min and treated with 1% SH and the mixture. Mild cytoplasmic vacuolations were histopathologically observed in the basal and wing cells in eyes desiccated for 60 min and treated with 1% SH and the mixture. The mixture solution containing 0.5% SH and 1% CO has protective effects against corneal desiccation similar to those of 1% SH and would be helpful as an artificial tear.

KEY WORDS: artificial tear, castor oil, porcine short-term dry eye model, sodium hyaluronate

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Artificial tears are essential agents for adjunctive therapy to lacrimostimulation in animals with tear film abnormalities, such as keratoconjunctivitis sicca (KCS) [9, 11, 12, 18, 20]. Unfortunately, lacrimomimetic therapy does not always achieve sufficient responses in spite of there being plenty of available tear substitutes [9, 11, 18], because frequent application of artificial tears, often more than 6 times per day, by the owners of animals with KCS is unattainable [9, 11, 18]. Owing to the lack of optimal artificial tears, many veterinarians may feel frustration when choosing artificial tears for lacrimomimetic therapy for KCS patients, suggesting that the prescription of artificial tears should be continuously improved in order to provide infrequent lacrimomimetic therapy to animals having tear film abnormalities.

Sodium hyaluronate (SH), which is a viscoelastic substance with mucinomimetic properties, has been used as a good corneal protectant in both humans and animals [1, 9, 11, 18, 20]. In addition, it is known that castor oil (CO) is one of the lipid products that not only improve stability, thickness and homogeneity of the tear film lipid layer, but also inhibit evaporation of aqueous tear from the corneal surface [10, 14, 16]. Hence, the combination of SH and CO may be an effective prescription as an artificial tear substitute for animals with tear film abnormalities.

The corneal-protective effects of newly developed artificial tears are generally assessed with *in vivo* dry eye models using live experimental animals, such as rabbits [3, 6, 7], although the animals are exposed to unexpected adverse effects and/or inefficacies of the new agents, thereby suffering pain associated with corneal damage induced by the newly developed artificial tears [7]. Hence, we believe that the corneal-protective effects of new artificial tears should be initially evaluated with an *in vitro* model to minimize the use of live experimental animals. The aims of this study are thus 1) to establish an *in vitro* porcine short-term dry eye model using a modified version of previous procedures [4, 6] and 2) to evaluate the corneal-protective effects of an artificial tear containing SH and CO by using this established dry eye model.

MATERIALS AND METHODS

Porcine eyes: Fresh enucleated porcine eyes were purchased from a local abattoir in Osaka, Osaka Meat and Organs Co., Ltd. The eyes with the surrounding eyelids and conjunctiva were immediately enucleated from slaughtered pigs, and the eyelids were closed to minimize corneal damage. The eyes were placed in a cool box kept at 4°C with moist conditions of saline (Otsuka Normal Saline, Otsuka, Tokyo, Japan) and transported to the laboratory at Osaka Prefecture University within an hour by motorcycle. Each porcine eye was rinsed with saline, and 1% (W/V) sodium

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Table 1. Kinetics of the degenerative changes of the cornea upon desiccation

Desiccation time (minutes)	Corneal staining score of methylene blue	Corneal absorbance at 660 nm of methylene blue	Corneal staining density of lissamine green
0	1.0 ± 0[a)]	0.017 ± 0.009[a)]	0.208 ± 0.019[a)]
60	2.4 ± 0.6[b)]	0.056 ± 0.033[a)]	0.268 ± 0.008[b)]
180	3.8 ± 0.5[c)]	0.206 ± 0.027[b)]	0.470 ± 0.016[c)]
360	4.0 ± 0[c)]	0.272 ± 0.053[b)]	0.612 ± 0.048[d)]

Different superscript letters indicate statistically significant differences between groups (P<0.05) (n=5).

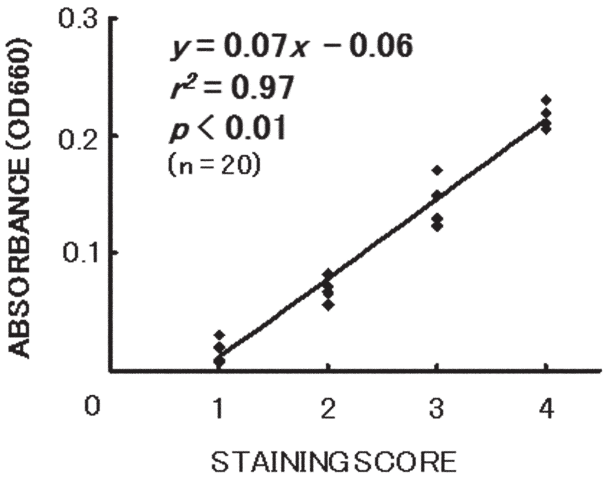

Fig. 1. Relationship between staining score of methylene blue (MB) and absorbance values of MB in the *in vitro* porcine short-term dry eye model. The solid line is the calculated regression.

fluorescein (SFL) solution prepared with saline and SFL powder (Nacalai Tesque, Kyoto, Japan) was applied to the porcine cornea in order to select porcine eyes with an intact cornea. The selected porcine eyes were gently rinsed with saline again, the eyelids were closed, and then, the eyes were again stored in the moist cool box (4°C) until the experiment, within approximately one hour.

Artificial tears: Four types of artificial tears were used in the experiments: 1) saline, 2) 0.1%, 0.5% or 1% (V/V) sodium hyaluronate (SH) solution prepared with saline and SH (Artz Dispo 25 mg, Seikagaku Corporation, Tokyo, Japan), 3) 0.5%, 1% or 5% (V/V) castor oil (CO) solution made with saline and CO (Sioe Pharmaceutical Co., Ltd., Amagasaki, Japan) and 4) a mixture solution of 0.5% (V/V) SH and 1% (V/V) CO. Each artificial tear was prepared before each experiment and stored in a sterilized eye dropper at room temperature (20–22°C). All eye droppers were shaken well just before application of the artificial tears to the porcine eyes.

Experimental procedures: An *in vitro* porcine dry eye model was designed by modification of the procedures of Choy *et al.* [4] and Fujihara *et al.* [6]. The eyelids of each porcine eye with an intact cornea were held open, excess saline was removed from the conjunctival sac with swabs, and then, the eyes were securely positioned on a plastic cap with the corneal surface up in the chamber kept at 20–22°C and 40–50% humidity without air flow in the room, with environmental conditions similar to those made with air-conditioners and dehumidifiers. In the first experiment on the *in vitro* porcine short-term dry eye model, the eyes treated without any artificial tears were desiccated for up to 360 min in the chamber described above. On the other hand, in the second experiment for evaluation of artificial tears including SH, CO and a mixture solution containing 0.5% SH and 1% CO, eyes were treated with 2 drops of each artificial tear (50 μl/drop) of saline, 0.1%, 0.5% or 1% SH solution, 0.5%, 1% or 5% CO solution or the mixture solution, blinked manually a couple of times by closing and opening the eyelids, held open again and then desiccated in the chamber for 60, 90 or 180 min. The control eyes were treated continuously with saline applied in the same chamber for the same desiccation time. In both experiments, all surrounding tissues of the eyes were removed after desiccation, and the corneas of the desiccated and control eyes 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 were used for staining dead and membrane-damaged cells of the cornea, respectively [6, 12, 22]. The stained eyes were washed well with saline to remove staining solutions, and the cornea stained with MB or LG was removed with surgical knives and scissors from the limbus of each eye. The corneas stained with MB were immediately photographed and placed in 2 m*l* of acetone/saturated sodium sulfate solution (volume ratio of 7:3) for 16 hr at room temperature (22°C) to extract MB. The photographed images were used for the evaluation of corneal staining scores indicating corneal integrity based on the extent of MB staining. The MB staining score was divided in 4 categories as follows: 1, less than 1/4 of the corneal area stained; 2, 1/4–1/2 of the corneal area stained; 3, 1/2–3/4 of the corneal area stained; and 4, more than 3/4 of the corneal area stained [6]. The absorbance of MB-extracted solutions was measured at 660 nm with a spectrophotometer (Smart Spec 3000, BIO-RAD, Hercules, CA, U.S.A.). The density of LG-stained corneas was measured using an image analyzer (FPD-100S, Fuji Film, Tokyo, Japan).

Histopathological examination: The harvested corneas from the desiccated and control eyes 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.

Statistical analysis: Values of the corneal staining scores

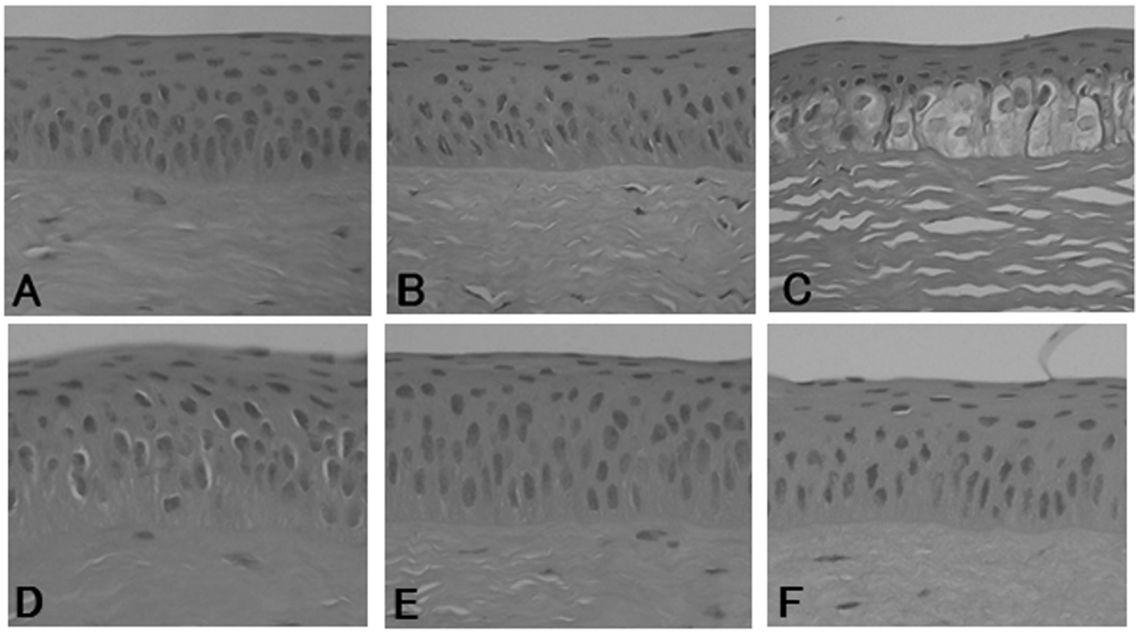

Fig. 2. Histopathologies of the desiccated cornea in the *in vitro* porcine short-term dry eye model. When compared with the cornea obtained immediately from the slaughtered animals (A), mild cytoplasmic vacuolations could be found in some basal cells of the cornea treated continuously with saline (B). The cornea desiccated for 180 min (C) had marked swollen basal cells with cytoplasmic vacuolations and nuclear swelling and wing cells with vacuole in the cytoplasm, and there were corneal basal and wing cells with mild to moderate cytoplasmic vacuolations on the cornea desiccated for 60 min (D). Meanwhile, histopathological alterations with cytoplasmic vacuolations were minimal on the corneal basal and wing cells in eyes desiccated for 60 min and treated with 1% SH (E) or a mixture solution containing 0.5% SH and 1% CO (F), when compared with saline non-treated eyes for 60 min of desiccation (D). × 400. HE staining.

of MB, the absorbance of MB extracted from the cornea and the corneal staining density of LG are shown as the mean ± standard deviation (SD), and their averages and SDs of continuous saline-treated control eyes were calculated from all of the control ocular data upon placement in the desiccated chamber for 60, 90 and 180 min, because the control data obtained from each time were similar. Pearson's correlation coefficients were calculated by the log-rank test. Data of artificial tears treated eyes were compared to the control values of ones treated continuously with saline, which were the group for which the eyes had similar conditions of normal eyes in the live animals. Comparison of values obtained in this experiment was carried out by using non-repeated measurement one-way analysis of variance (ANOVA) and then Scheffe's test or the Kruskal-Wallis H test, followed by the Mann-Whitney U-test with Bonferroni correction (Statcel 2nd ed.; OMS Publishing Co., Tokyo, Japan). A *P*-value less than 0.05 was considered to be statistically significant.

RESULTS

In vitro porcine short-term dry eye model: Corneal damage induced by desiccation was evaluated by the staining of non-viable cells, which were detectable using MB, and membrane-damaged cells, which were observable using LG. The corneal staining scores of MB, absorbance values of MB extracted from the corneas and values of the corneal staining density of LG increased significantly with increasing desiccation time (Table 1). In addition, a significant linear correlation was found between the staining score of MB and its absorbance (r^2=0.97, P<0.01); the regression equation between these 2 variables was y=0.07 x − 0.06 (Fig. 1). Mild cytoplasmic vacuolations were found in some basal cells on the control cornea treated continuously with saline when compared with the cornea harvested immediately from the slaughtered animals (Fig. 2A and 2B), whereas conspicuous alterations could be observed in the histopathology of cornea desiccated for 180 min. The cornea desiccated for 180 min had not only marked swollen basal cells with cytoplasmic vacuolation and nuclear swelling, but also wing cells with vacuole in the cytoplasm (Fig. 2C).

Corneal-protective effects of artificial tears on the porcine short-term dry eye model: Table 2 shows the results of MB and LG staining in eyes treated with saline, SH solutions, CO solutions and a mixture solution containing 0.5% SH and 1% CO on the porcine short-term dry eye model. All control eyes with continuous treatment of saline (CTS) for 60, 90 and 180 min showed similar results of MB and LG staining, and their minimal corneal damage was demonstrated by their staining, as shown in Table 2, when compared with the ocular group indicated as 0 min of desiccation time in Table 1 of the *in vitro* porcine short-term dry eye model, which was

Table 2. Corneal-protective effects of artificial tears of sodium hyaluronate, alone castor oil alone, and a mixture containing sodium hyaluronate and castor oil

Desiccation time (minutes)	Control	SH			CO			MIXTURE
		1%	0.5%	0.1%	5%	1%	0.5%	0.5%SH+1%CO
Corneal staining score of methylene blue								
0 (CTS)	1.0 ± 0	–	–	–	–	–	–	–
60	(3.3 ± 0.6)	1.1 ± 0.3*	2.0 ± 0.5	2.7 ± 0.5	2.2 ± 0.7	3.2 ± 0.7	3.3 ± 0.5	1.0 ± 0*
90	(3.7 ± 0.6)	1.4 ± 0.5*	2.3 ± 0.5	3.0 ± 0	2.9 ± 0.3	3.7 ± 0.5	3.9 ± 0.3	1.8 ± 0.4
180	(4.0 ± 0)	2.0 ± 0.7	2.9 ± 0.3	3.8 ± 0.4	3.6 ± 0.5	3.9 ± 0.3	4.0 ± 0	2.1 ± 0.3
Corneal absorbance at 660 nm of methylene blue								
0 (CTS)	0.048 ± 0.008	–	–	–	–	–	–	–
60	(0.163 ± 0.021)	0.051 ± 0.020*	0.096 ± 0.045	0.113 ± 0.075	0.090 ± 0.011	0.114 ± 0.021	0.175 ± 0.061	0.058 ± 0.006*
90	(0.182 ± 0.017)	0.074 ± 0.034*	0.114 ± 0.069	0.132 ± 0.089	0.091 ± 0.037	0.115 ± 0.043	0.176 ± 0.070	0.076 ± 0.007
180	(0.205 ± 0.031)	0.162 ± 0.074	0.126 ± 0.062	0.151 ± 0.034	0.147 ± 0.047	0.134 ± 0.016	0.196 ± 0.077	0.113 ± 0.013
Corneal staining density of lissamine green								
0 (CTS)	0.219 ± 0.015	–	–	–	–	–	–	–
60	(0.340 ± 0.046)	0.232 ± 0.010*	0.262 ± 0.023	0.307 ± 0.023	0.293 ± 0.050	0.345 ± 0.021	0.340 ± 0.073	0.221 ± 0.026*
90	(0.393 ± 0.040)	0.267 ± 0.036	0.301 ± 0.029	0.330 ± 0.025	0.328 ± 0.058	0.380 ± 0.024	0.364 ± 0.071	0.252 ± 0.021
180	(0.430 ± 0.030)	0.317 ± 0.023	0.326 ± 0.041	0.352 ± 0.034	0.361 ± 0.079	0.426 ± 0.019	0.413 ± 0.076	0.289 ± 0.018

CTS: Continuous treatment with saline, SH: Sodium hyaluronate, CO: Castor oil. A superscript asterisk indicates no significant differences between control (CTS; 0 min of desiccation time) and each experimental group. n=8–9 (Values with parentheses were calculated from the data of 3 eyes treated with saline).

the group for which the eyes were stained with the dyes at the earliest time after enucleation. There were no significant differences between eyes treated continuously with saline (control) and those desiccated for 60 and 90 min and treated with 1% SH in terms of the staining scores and absorbance values of MB. No significant difference of the staining density of LG was only observed between control eyes and 1% SH-treated ones with 60 min of desiccation. Histopathological changes with cytoplasmic vacuolation were minimal on the corneal basal and wing cells in eyes desiccated for 60 min with 1% SH treatment, when compared with saline non-treated eyes for 60 min of desiccation (Fig. 2D and 2E). Significant differences were found in the staining scores of MB and absorbance values of MB between control and 1% SH-treated eyes for 180 min of desiccation and in the values of the staining density of LG between control and 1% SH-treated eyes for 90 and 180 min of desiccation (Table 2). There were no significant differences between control eyes and those desiccated for 60 min with the treatment of the mixture solution in terms of the staining scores of MB, absorbance values of MB and values of the staining density of LG (Table 2), indicating that corneal protection similar to that for 1% SH-administered eyes for 60 min of desiccation was obtained in the eyes treated with the mixture solution containing 0.5% SH and 1% CO. As shown in Fig. 2E and 2F, the cornea desiccated for 60 min and treated with the mixture solution had minimal histopathological changes on the basal and wing cells, which were similar to the changes of 1% SH-treated eyes with 60 min of desiccation. Unfortunately, significant differences were observed in the results of MB and LG staining between the control and eyes treated with the mixture solution for 90 and 180 min desiccation time. There were significant differences in the staining results of MB and LG between the control and eyes treated with solution of 0.5% SH, 0.1% SH, 5% CO, 1% CO and 0.5% CO for all desiccation times (Table 2).

DISCUSSION

An *in vitro* porcine short-term dry eye model was established in this study. Three different staining dyes, SFL, MB and LG, were used in this model. MB and LG were selected for the detection of non-viable dead cells and membrane-damaged cells of the corneal epithelium, respectively [6, 12, 22]. SFL was employed to select eyes with an intact cornea without corneal defects, because the dye did not adhere to an intact epithelial surface [12]. If MB or LG was used for ocular selection and the dye was left on the corneal surface, overestimated results might arise due to the remaining dye used at the time of the selection. However, SFL only stains the disrupted part of the corneal epithelium lightly, if there is little intercellular space on the cornea [12]. The stained parts of SFL would be overlaid with dark dye of MB or LB, resulting in no influence on the evaluation system with MB and LG in the dry eye model. The stainabilities of MB and LG significantly increased with increasing desiccation time in the dry eye model (Table 1), and there was a linear correlation between the staining score of MB, as a qualitative evaluation, and the absorbance of MB, as a quantitative analysis, using a spectrophotometer (Fig. 1). These results are similar to the findings observed in an *in vivo* rabbit short-term dry eye model reported previously [6]. The histopathological alterations observed in the desiccated porcine cornea were very similar to the alterations found in a mouse model of dry eye and experimental canine KCS [5, 8]. Moreover, both MB and LG staining was related to the degree of histopathological change in this study (Table 1 and Fig. 2A–2C). All these findings suggest that the porcine short-term dry eye

model can reflect the corneal changes upon desiccation in live animals and is available for evaluating the potencies for corneal protection of newly developed artificial tear substitutes by *in vitro* studies. The dry eye model should have the following advantages: 1) not only preventing exposure to unexpected adverse effects and/or inefficacies of the newly developed artificial tears, but also reducing the number of experimental animals in studies dealing with assessment of potential therapeutic agents [13], 2) being able to collect a large amount of data on novel artificial tears in a short period [6] and 3) rapidly assessing the therapeutic effects of new artificial tears [6]. On the other hand, the dry eye model cannot evaluate phenomena exhibited by live animals, such as blinking and corneal wound healing [2, 17, 19], and cannot evaluate long-term and adverse reactions of newly developed artificial tears, because it uses enucleated eyes, which exhibit postmortem changes. Therefore, the porcine short-term dry eye model established in this study is useful for screening in the selection of potential agents before experiments with live animals.

There are plenty of artificial tear substitutes for lacrimomimetic therapy in animals with an absence or a reduction of lacrimal secretions, and they generally contain one or more ingredients that can replace deficiencies of tear components [9, 11]. It is well known that SH has mucinomimetic properties with viscosity and wettability and is a useful corneal protectant in animals with KCS [9, 11, 15, 18, 20, 23]. Judging from our results, 1% SH may be available as an artificial tear. However, the clinical usefulness of 1% SH would be low, because it is an expensive agent and may have a disadvantage of provoking an uncomfortable sensation when the drug is applied to the eyes, due to its high viscosity [11, 12, 18]. The concentrations of commercially available ophthalmic SH solutions are 0.1% and 0.3% in Japan [21] and 0.15% and 0.4% in other countries [9, 11, 18, 23]. However, it is still difficult to provide infrequent lacrimomimetic therapy, fewer than 6 times per day, to animals with tear film abnormalities in spite of the presence of good ophthalmic SH solutions [9, 11, 18]. This is not surprising, because sufficient corneal-protective effects could not be found in eyes treated with 0.5% SH, which was a greater concentration than that in commercially available ophthalmic SH solutions, in the porcine short-term dry eye model (Table 2). Natural tear film consists of 3 components: lipid, an aqueous component and mucin, and the layer containing lipid inhibits the evaporation of the aqueous component from tear film [12, 14, 16, 18, 20]. Hence, insufficient corneal protection should be attributable to a lack of an effect inhibiting the evaporation of water from SH. A surface coating layer could be added to inhibit such evaporation, thereby improving SH ophthalmic solution to achieve infrequent lacrimomimetic therapy in animals.

Lipid products or oils are generally used as ingredients of ophthalmic ointments [9, 11], although the solution type of artificial tear containing lipid products, such as CO, was shown to be helpful for treating patients with tear film abnormalities or dry eye model animals [10, 14, 16]. In addition, CO was shown to inhibit the evaporation of water from the corneal surface [14, 16]. Therefore, combined prescription of saline, SH and CO, which is similar to natural tear film containing lipid, an aqueous component, and mucin [11, 18, 20], was established and assessed in the experiment. The saline-based tear substitute containing 0.5% SH and 1% CO showed corneal-protective effects equal to those with 1% SH (Table 2), indicating that this combination of an aqueous component (saline), mucinomimetics (SH) and lipid (CO) is an effective prescription for an artificial tear substitute for treating cases with tear film abnormalities in animals. However, the maximum time of corneal protection was only 60 min for both 1% SH and the mixture solution containing 0.5% SH and 1% CO in the porcine short-term dry eye model, when compared with the group having continuous treatment of saline (Table 2). This corneal-protective time would still be too short for providing infrequent applications of artificial tears in animals with KCS. The prescription of our artificial tear substitute presented here should be improved to achieve infrequent lacrimomimetic therapy of fewer than 6 times per day in cases with tear film abnormalities. In order to determine the appropriate prescription for long-acting artificial tear substitutes, more detailed prescriptions should be studied or different types of SH and/or lipid products should be used in the future.

In summary, the saline-based tear substitute containing 0.5% SH and 1% CO had protective effects against corneal desiccation similar to those of 1% SH for 60 min. The combined prescription of saline, 0.5% SH and 1% CO would be useful as an artificial tear substitute for treating animals with tear film abnormalities, such as KCS.

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