Supercritical fluid-mediated liposomes containing cyclosporin A for the treatment of dry eye syndrome in a rabbit model: comparative study with the conventional cyclosporin A emulsion

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Background: The objective of this study was to compare the efficacy of cyclosporin (CsA)-encapsulated liposomes with the commercially available CsA emulsion (Restasis®) for the treatment of dry eye syndrome in rabbits.

Methods: Liposomes containing CsA were prepared by the supercritical fluid (SCF) method consisted of phosphatidylcholine from soybean (SCF-S100) and egg lecithins (SCF-EPCS). An in vitro permeation study was carried out using artificial cellulose membrane in Franz diffusion cells. Dry eye syndrome was induced in male albino rabbits and further subdivided into untreated, Restasis®-treated, EPCS, and S100-treated groups. Tear formation in the dry-eye-induced rabbits was evaluated using the Schirmer tear test. All formulations were also evaluated by ocular irritation tests using the Draize eye and winking methods with the determination of CsA concentration in rabbit tears.

Results: After the treatment, the Schirmer tear test value significantly improved in EPCS-treated (P=0.005) and S100-treated (P=0.018) groups compared to the Restasis®-treated group. The AUC0–24 h for rabbit’s tear film after the administration of SCF-S100 was 32.75±9.21 μg⋅h/mg which was significantly higher than that of 24.59±8.69 μg⋅h/mg reported with Restasis®. Liposomal CsA formulations used in this study showed lower irritation in rabbit eyes compared with Restasis®.

Conclusion: These results demonstrate that the novel SCF-mediated liposomal CsA promises a significant improvement in overcoming the challenges associated with the treatment of dry eyes.

Keywords: cyclosporin A, liposomes, supercritical fluid, dry eye syndrome

Introduction
Dry eye syndrome (DES) is a common ophthalmic condition, which is defined as a disorder of the tear film due to tear deficiency or excessive evaporation.1 DES causes damage to the interpalpebral ocular surface that is associated with symptoms of discomfort.2 It is believed to affect millions of people worldwide.3 The use of topical steroids has been shown to produce relief from dry eye symptoms, but long-term use results in potential side effects such as glaucoma and cataract.4 Therefore, the development of agents is essential for chronic use of the drug without serious adverse effects.

Cyclosporin A (CsA), a neutral and hydrophobic cyclic peptide of eleven amino acids,5 is isolated from a fungus Tolypocladium inflatum Gams.6 The mechanism of action of how CsA increases tear production is still unknown, but it seems to be related...
to its immunomodulatory activity, which decreases local inflammation.\textsuperscript{1} In the cytoplasm, CsA binds to its immunophilin, cyclophilin, forming a complex and the complex then binds and blocks the function of the enzyme calcineurin that results in the suppression of the expression of cytokine genes and thereby inhibits T-cell activation.\textsuperscript{6,8,9} CsA has been shown to be an effective therapeutic agent for treatment of dry eye symptoms,\textsuperscript{3,10} leading to approval of an oil-in-water 0.05% CsA emulsion (Restasis\textsuperscript{®}; Allergan Inc., Irvine, CA, USA) in December 2002.\textsuperscript{8} Restasis\textsuperscript{®} formulation contains castor oil, of which the most common side effect is ocular burning or irritation following the long-term use of Restasis.\textsuperscript{9,11} In addition, adverse reactions such as itching, redness, visual disturbance, and eye pain have limited its use.\textsuperscript{12} Poor ocular tolerance, low bioavailability, and instability are also the major drawbacks reported with Restasis.\textsuperscript{8,11}

Over the past few decades, a number of studies have focused on the potential application of liposomes as alternatives to the conventional therapy of CsA.\textsuperscript{5,11–16} In addition, liposomal technology has also been studied for the ocular delivery of CsA,\textsuperscript{17–19} but the large-scale manufacture of these liposomes is expensive and challenging. Liposomes were reported to enhance the permeation of poorly adsorbed drug by binding to the corneal surface and improving residence time.\textsuperscript{20} These liposomes could be used to improve the pharmacokinetic profile, enhance therapeutic effect, and reduce toxicity associated with higher doses.\textsuperscript{21} Despite these advantages, no studies have been published regarding the use of liposomal CsA in the treatment of DES.

Large-scale production and stability issues of liposomes are major hurdles to overcome in using liposomes as candidates for CsA delivery.\textsuperscript{22,23} In our previous study,\textsuperscript{5} we reported the novel formulations of liposomal CsA using the supercritical fluid of carbon dioxide (SCF-CO\textsubscript{2}) method that could address the issues of scaled-up production and stability of liposomes. In the present study, we prepared liposomal formulations of CsA using phosphatidylcholine from soybean (SCF-S100) and egg lecithins (SCF-EPCS) based on the SCF-CO\textsubscript{2} method. Both of these liposomes were easy to manufacture and require low-cost of production. The aim of this study was to investigate the effectiveness of SCF-mediated liposomal CsA for the treatment of DES in rabbits compared with conventional CsA emulsion, Restasis\textsuperscript{®}. The efficiency of SCF-mediated CsA liposomes in producing tears was tested in DES-induced rabbit eyes by analyzing the changes in the Schirmer tear test (STT). The ocular irritation tests and concentration determination of CsA in tear films were also carried out to investigate the strength of newly proposed liposomal formulations.

### Materials and methods

#### Materials

Restasis\textsuperscript{®} emulsion (Allergan Inc.) was purchased from a local market. CsA was supplied from Concord Drugs Limited (Hyderabad, India). Ethyl alcohol (purity 99.9%) and methyl alcohol (purity 99.5%) were purchased from Samchun Pure Chemical Co. Ltd. (Gyeonggi-do, Republic of Korea). A dialysis membrane (molecular weight cut-off: 14,000 Dalton (Da)) from Sigma-Aldrich Co. (St Louis, MO, USA) was used. Purified water of Milli-Q quality (Milli-Q Reference; EMD Millipore, Billerica, MA, USA) was used throughout the study.

#### Preparation of CsA-containing liposomes

Liposomes were prepared using the SCF-CO\textsubscript{2} method described in our previous reports.\textsuperscript{5,24} Two different formulations of CsA SCF-S100 and SCF-EPCS liposomes containing naturally derived phosphatidylcholine from soybean (Lipoid S100; Lipoid GmbH, Ludwigshafen, Germany)\textsuperscript{3} and egg lecithin (Lipoid EPCS; Lipoid GmbH)\textsuperscript{24} were used in this study. In brief, 200 mg of phospholipid, 100 mg of cholesterol, and 50 mg of CsA were dissolved in ethanol and the mixture was then sealed in a reaction vessel with 900 mg of anhydrous lactose powder. The supercritical CO\textsubscript{2} was pumped to the vessel at an optimized temperature of 45°C and pressure of 10 MPa. A thin film of CsA-phospholipid mixture coated on the surface of lactose was formed upon depressurization of the vessel. The resulting thin film was then hydrated at 50°C to form multimellar liposomes. Prepared liposomes were homogenized by passing through an ultra-high-pressure homogenizer (Nano DeBEE; BEE International, South Easton, MA, USA) to obtain the nano-sized liposomes. An operating pressure of 30,000 psi and nine numbers of run were selected based on the previous optimization study.\textsuperscript{24}

#### Particle size and polydispersity index (PDI) determination

Both SCF-S100 and SCF-EPCS liposomes were properly diluted with filtered Milli-Q water (0.45 μm pore size polytetrafluoroethylene Whatman\textsuperscript{24} filters; GE Healthcare Life Sciences, Pittsburgh, PA, USA) before measurements to adjust the intensity. The particle size and PDI of liposomes and Restasis\textsuperscript{®} were measured using a particle size analyzer (ELS-Z; Otsuka Electronics Co., Ltd., Osaka, Japan) at room temperature. The PDI was determined as a measure of the level of homogeneity of particle sizes and a PDI <0.1 represented monodispersion, while PDI >0.1 represented polydispersion of liposomal vesicles.\textsuperscript{5}
Scanning electron microscopy (SEM) of liposomes

SCF-S100 liposomes were freeze-dried using a laboratory freeze-dryer (FD 5512; Ilshin Lab Co. Ltd., Gyeonggi-do, Republic of Korea) in order to perform SEM. The morphology of anhydrous lactose powder and CsA entrapped SCF-S100 liposomes was investigated by Field Emission-SEM (JSM-7100F, JEOL, Tokyo, Japan) at an accelerating voltage of 5.0 kV. The samples were prepared on aluminum stubs and coated with gold under argon atmosphere by means of a sputter coater.

High-performance liquid chromatography (HPLC) analysis of CsA

CsA was analyzed by the HPLC method explained in Karn et al.3 In brief, homogenized liposomes were appropriately diluted and dispersed with ultra-pure water filtered with a 0.45 μm polystyrene fluoroethylene syringe filter and centrifuged to separate free drug. After centrifugation, the supernatants were discarded and the pellets were dissolved with ultra-pure filtered water to make 0.05% CsA. Samples were directly assayed by HPLC (Agilent 1200 Series System; Agilent Technologies, Santa Clara, CA, USA) using a C18 analytical column (4.6 mm × 150 mm, 3 μm; SUPELCO Analytical, Bellafonte, CA, USA) at 70°C. The mobile phase consisted of acetonitrile and distilled water (90%;10%, volume/volume) at a flow rate of 1.0 mL/minute. Sample injection volumes were 10 μL, and CsA detection was performed using an ultraviolet (UV) detector (Agilent Technologies) at a wavelength of 210 nm. The concentration of CsA in liposomal samples to be used for the in vivo study and Restasis® emulsion was the same, and 50 μL of each contained 25 μg of CsA.

In vitro permeation studies in Franz diffusion cells

The permeation studies were performed using Franz diffusion cells (transdermal diffusion cell drive console with tilt; Logan Instruments Corp., Franklin Township, NJ, USA) with an orifice size of 5 mm and area of 0.20 cm² as described by Aksungur et al.12 Dialysis tubing cellulose membrane (D9777-100 FT; Sigma-Aldrich Co.) with a molecular weight cut-off of 14,000 Da was used. Membranes were soaked in Milli-Q water for 24 hours before mounting in Franz diffusion cells. The simulated lacrimal fluid (SLF) was prepared using 8.3 g of NaCl, 0.084 g of CaCl₂·2H₂O, and 1.4 g of KCl in 1 L of Milli-Q water; 10 mL of SLF was placed in each of six receiver compartments of the Franz diffusion cell and temperature was maintained at 32°C, which is based on the temperature of the eye surface. The stirring rate (300 rpm) was constant throughout the study. The total content of CsA in Restasis® and CsA-encapsulated liposomes was assayed using HPLC before applying to the donor compartment. A volume of 2 mL of each sample was gently placed into the donor compartment. At specified time intervals, 1 mL samples were withdrawn from the receiver compartment for HPLC determination and replaced immediately with an equal volume of fresh SLF solution.

In vivo study of CsA liposomes in DES-induced rabbit model

Animal handling

Male New Zealand albino rabbits aged 9 weeks, weighing 1.5–2.0 kg (Somatco Inc., Seoul, Republic of Korea) were used in this study. All experimental procedures for animal studies were approved by the Committee for the Care and Use of Laboratory Animals, Yonsei University and performed in accordance with the Committee’s Guidelines and Regulations for Animal Care. All rabbits were individually housed in separate cages under standardized temperature (19°C±1°C), humidity (50%–60%), and light (12-hour light-dark cycle) conditions. They were fed the standard diet (Somatco Inc.) and tap water.

Induction of DES and the STT

Fifteen male albino rabbits were divided into control and four different DES-induced groups, each group consisting of three rabbits. Only 0.9% (volume/volume) NaCl was applied to both eyes of rabbits of the control group. To induce dry eye, 1% atropine sulfate (AS; Sigma-Aldrich Co.) was instilled into both eyes of rabbits of the four DES-induced groups three times a day for 10 days. Further, dry-eye-induced groups were divided into untreated, Restasis®-treated, SCF-EPSCs-liposomes-treated (EPCS-treated), and SCF-S100-liposomes-treated (S100-treated) groups.

The STT without anesthesia (type I) was performed to measure the aqueous portion of tear secretion in response to both conjunctival stimulation and basal non-reflex secretion.25,26 For the first 3 days, only 50 μL of 1% AS was instilled into both eyes of all 12 rabbits to induce DES. From the fourth day onwards, 0.5 hours after instillation of 1% AS, 50 μL of Restasis®, SCF-EPSCs liposomes, and SCF-S100 liposomes were also administered into both eyes of Restasis®-treated, EPCS-treated, and S100-treated groups, respectively. The Schirmer test was performed at the beginning of this experiment and on day 3, 5, 7, and 10. The test was performed using ColorBar™ STTs (Eagle Vision,
Memphis, TN, USA), which were made of 5×35 mm standard-
dized filter paper strips with a blue color bar that moves
with a tear front. The strip was placed inside the margin of
the inferolateral third of the lower eyelid for two minutes and
the level of strip wetting was measured after a few minutes.
The height of the Schirmer test strips wetted by the tears was
measured in millimeters and plotted versus time.

CsA determination in rabbit tears
The concentration of CsA in rabbit eyes was determined by
the modified method of Aksungur et al. A total of 12 male
New Zealand white rabbits separated into groups 1, 2, and
3 were used for this study. Group 1 was treated with a Resta-
sis® emulsion, whereas groups 2 and 3 were treated with
SCF-S100 liposomes and SCF-EP-CS liposomes, respectively.
A single instillation of 100 μL of drug was used on the left eye,
and the untreated contralateral eye was used as the control into
which 0.9% (weight/volume) NaCl solution was administered.
Tear fluid samples were collected with Schirmer filter papers,
which were weighed before use and gently inserted into the
inferolateral cul-de-sac of the eye, close to the middle of the
eye, for 30 seconds. Immediately after collection; papers were
removed, placed into the Eppendorf tubes, and weighed again.
Papers were then placed in 300 μL of methanol, vortexed for
3 minutes, and centrifuged (1730R; LaboGene, Seoul, Republic
of Korea) at 14,000 rpm for 15 minutes. After that, CsA content
in each sample was determined by HPLC assay.

Ocular irritation tests
Ocular irritation tests were used to evaluate the ocular toler-
ance of liposomal formulations. These tests were performed
using the Draize eye and winking tests. For the Draize eye
test, eight male albino rabbits were used. For acute irrita-
tion, three rabbits received three consecutive instillations of
50 μL of SCF-S100 liposomes into the lower conjunctival
sac with 10-minute intervals, and 30 minutes after the treat-
mant, rabbits were examined for signs of ocular irritation. For
long-term irritation, three rabbits received instillations three
times a day for 7 days, and the rabbits were examined at the
end of the treatment. Untreated rabbits were used as a con-
control. The animals’ discomfort and symptoms and signs in the
conjunctiva, cornea, and lids were macroscopically examined
using the Acute Eye Irritation/Corrosion scoring system estab-
lished by the 2012 Organization for Economic Cooperation and
Development for ocular irritation testing (Table 1). 27–30

The ocular irritation test was also evaluated by the rabbit
winking method described by Li et al. In this method, eight
male albino rabbits were divided into two groups: Restasis®
treated and liposome-treated groups. For all rabbits, 50 μL of
normal saline was instilled into the right eyes, whereas 50 μL
of Restasis® in Restasis®-treated group and SCF-S100 lipo-
some in SCF-S100-liposomes-treated group were instilled
gently into the left eyes. After instillation, drugs were spread
uniformly on the eye surface. Ten seconds after the administra-
tion of the drop to the eyes, the frequency of the winking of the
rabbits, for 2 minutes, was recorded. The winking of the right
eye was used as a control to compare the irritation effect.

Statistical analysis
Statistical analysis was performed using SPSS software,
version 18.0 for Windows (IBM Corporation, Armonk,
NY, USA). Independent sample Student’s t-tests (assuming
unequal variance) were used to compare the mean values
between the two formulations, whereas the one-way analy-
asis of variance (ANOVA) test followed by Tukey’s honest
significant difference test and Student–Newman–Keuls tests
were performed to demonstrate the statistical differences
among groups. The level of significance was set as a P-value
of less than 0.05.

Results and discussion
Preparation and characterization
of SCF-mediated liposomes
Synthesized SCF-S100 and SCF-EP-CS liposomes contained
phosphatidylcholine (Lipoid S100 or EPCS), cholesterol,
CsA, and anhydrous lactose. The initial size of SCF-S100 and
SCF-EP-CS liposomes was 1138±120 nm and 1012±170 nm,
respectively. The liposomes were homogenized at 30,000 psi

Table 1 Grading of ocular irritation test

<table>
<thead>
<tr>
<th>Grade</th>
<th>Discomfort</th>
<th>Cornea</th>
<th>Conjunctiva</th>
<th>Discharge</th>
<th>Lids</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No reaction</td>
<td>No alteration</td>
<td>No alterations</td>
<td>No discharge</td>
<td>No swelling</td>
</tr>
<tr>
<td>1</td>
<td>Blinks</td>
<td>Mild opacity</td>
<td>Mild hyperemia; mild edema</td>
<td>Mild discharge without moistened hair</td>
<td>Mild swelling</td>
</tr>
<tr>
<td>2</td>
<td>Enhanced blinking; intense tearing; vocalizations</td>
<td>Intense opacity</td>
<td>Intense hyperemia; intense edema; hemorrhage</td>
<td>Intense discharge with moistened hair</td>
<td>Obvious swelling</td>
</tr>
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</table>
to produce a size of 146±28 nm and 148±24 nm, respectively. The PDI of homogenized SCF-S100 and SCF-EPCS liposomes was 0.14±0.06 and 0.13±0.05, respectively, whereas the size and PDI of Restasis® used in this experiment were 317.6±9.3 nm and 0.32±0.02, respectively. The small PDIs of SCF-mediated liposomes suggested a more homogeneous vesicle population compared with conventional CsA emulsion, Restasis®. We obtained nanosized liposomes after the high-pressurized homogenization of initial SCF-S100 and SCF-EPCS liposomes, which were used for ocular delivery of CsA in this study.

The application of liposomes in ophthalmic drug delivery is immense; however, none of the liposomal drugs have been approved for ocular administration. Despite liposomes’ demonstrated improvements in precorneal retention, sustained drug release, and transcorneal permeation, they still face challenges in their limited long-term physical stability and drug-loading capacity due to the inherent complex nature of their structures. Other major reasons are limited clinical and scientific results obtained with ophthalmic liposomes and difficulty in the large-scale synthesis procedure. All the major marketed liposomal drugs require high-grade synthetic saturated phospholipids and complex manufacturing procedures for their synthesis. Consequently, the production cost of large-scale production of liposomal drugs turns out to be very high compared with conventional drugs.

Proposed liposomal formulations in this study need a simple experimental setup with low capital cost, and naturally derived phospholipids that are much cheaper than synthetic phospholipids. Although we are using low-cost phospholipids (Lipoid S100 and Lipoid EPCS), the physicochemical properties of liposomes are not compromised. From the current study, we report a large-scale production of liposomal products of CsA that are cost-effective and easy to manufacture. These novel CsA formulations were nanosize-based preparations for the enhanced ocular delivery potential of CsA.

SEM images of SCF-mediated liposomes

The SEM photographs of lactose powder and SCF-S100 liposomes are shown in Figure 1. The spherical vesicular structure of SCF-S100 liposomes (Figure 1A and Figure 1B) could be easily identified compared with lactose powder (Figure 1C), which was identical to a previous transmission electron microscopy.
report of CsA-liposomes.\textsuperscript{5} The particle size of SCF-S100 liposomes was about 100–200 nm, which was in good correlation with results obtained by particle size measurement.

**In vitro permeation studies**

The results of the in vitro permeation test are shown in Figure 2. The purpose of this study was to obtain pre-information about in vivo studies by using artificial cellulose membrane simulating human cornea.

The cumulative percentage of CsA diffused in the receptor compartment was 33.76 with Restasis\textsuperscript{®} and 31.32 with SCF-S100 liposomes after 24 hours. No significant difference between the developed liposomal formulation and marketed Restasis\textsuperscript{®} in release and diffusion through the membrane, was observed.

**STT**

The STT values of the control group did not show any noticeable and significant changes of the tear volume production during the 10 days of in vivo experimentation. The STT value of the control group was $15.50 \pm 3.69$ mm before starting the experiment (day 0), and $15.75 \pm 3.93$ mm ($P=0.907$) at the end of the experiment.

Table 2 summarizes STT values of all four dry-eye-induced groups. On day 0, the STT values of all groups were significantly similar (ANOVA, $P=0.498$) and values were around $15 \text{ mm}$, which is considered a normal value.

<table>
<thead>
<tr>
<th>Days</th>
<th>Untreated</th>
<th>Restasis\textsuperscript{®}-treated</th>
<th>EPCS-treated\textsuperscript{1}</th>
<th>SCF-S100-treated\textsuperscript{2}</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>13.08±1.56</td>
<td>15.08±4.16</td>
<td>14.50±0.70</td>
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<td></td>
<td>$P_{1}$</td>
<td>0.31</td>
<td>0.082</td>
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<td></td>
<td>$P_{2}$</td>
<td></td>
<td>0.748</td>
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</tr>
<tr>
<td></td>
<td>$P_{3}$</td>
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<td>0.68</td>
<td>0.686</td>
</tr>
<tr>
<td></td>
<td>$P_{4}$</td>
<td></td>
<td>0.498</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>9.75±2.31</td>
<td>8.46±3.77</td>
<td>9.50±2.36</td>
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<tr>
<td></td>
<td>$P_{1}$</td>
<td>0.064</td>
<td>0.468</td>
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</tr>
<tr>
<td></td>
<td>$P_{2}$</td>
<td></td>
<td>0.163</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>$P_{3}$</td>
<td></td>
<td></td>
<td>0.714</td>
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<tr>
<td></td>
<td>$P_{4}$</td>
<td></td>
<td></td>
<td>0.823</td>
</tr>
<tr>
<td>5</td>
<td>9.0±2.16</td>
<td>11.2±1.21</td>
<td>13.16±2.38</td>
<td>13.83±2.25</td>
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<tr>
<td></td>
<td>$P_{1}$</td>
<td>0.025\textsuperscript{a}</td>
<td>0.015\textsuperscript{a}</td>
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<td></td>
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<td>0.119</td>
<td>0.039\textsuperscript{a}</td>
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<td></td>
<td>$P_{3}$</td>
<td></td>
<td></td>
<td>0.628</td>
</tr>
<tr>
<td></td>
<td>$P_{4}$</td>
<td></td>
<td></td>
<td>0.002\textsuperscript{a}</td>
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<tr>
<td>8</td>
<td>8.75±0.97</td>
<td>12.0±3.40</td>
<td>14.08±3.82</td>
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<tr>
<td></td>
<td>$P_{1}$</td>
<td>0.071</td>
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<tr>
<td></td>
<td>$P_{2}$</td>
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<td></td>
<td>$P_{3}$</td>
<td></td>
<td></td>
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</tr>
<tr>
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<td>$P_{4}$</td>
<td></td>
<td></td>
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<tr>
<td>10</td>
<td>8.10±2.13</td>
<td>11.75±1.54</td>
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<td>14.91±2.20</td>
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<td>$P_{4}$</td>
<td></td>
<td></td>
<td>0.000\textsuperscript{a}</td>
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</table>

Notes: Each group consists of three rabbits with a total number of six eyes. Data are expressed as mean ± standard deviation. \textsuperscript{a}Significant difference when $P<0.05$. SCF-EPCS liposomes. \textsuperscript{5}SCF-S100 liposomes. \textsuperscript{P_{1}}Restasis\textsuperscript{®}-treated, EPCS-treated, and S100-treated groups are compared with the untreated group. \textsuperscript{P_{2}}EPCS-treated and S100-treated groups are compared with the Restasis\textsuperscript{®}-treated group. \textsuperscript{P_{3}}S100-treated group is compared with the EPCS-treated group. \textsuperscript{P_{4}}one-way ANOVA between the four groups. Restasis\textsuperscript{®}, Allergan Inc., Irvine, CA, USA.

Abbreviations: ANOVA, analysis of variance; SCF-S100, supercritical fluid-mediated liposomes prepared using Lipoid S100; SCF, supercritical fluid; S100, phosphatidylcholine from soybean lecithin; EPCS, phosphatidylcholine from egg lecithin; SCF-EPCS, supercritical fluid-mediated liposomes prepared using Lipoid EPCS.
A significant decrease in the tear production values was observed after the 3 days of 1% AS administration. Following treatment with drugs of only 2 days, a noticeable change appeared, and STT values were significantly increased for all treated groups. Interestingly, the STT values of groups treated with the liposomal formulation reached almost normal values even after 2 days of drug treatment. Compared with the conventional emulsion (Restasis®), the liposomal formulations showed an improvement as demonstrated by the difference of the STT values between day 5 and day 0 (3.83 for Restasis®, P=0.090; 1.33 for SCF-EPSC liposomes, P=0.267; and 0.50 for S100 liposomes, P=0.646). After treatment for 7 days (on day 10), there was a significant improvement (P=0.007) in the STT values of the Restasis®-treated group compared with the untreated group. However, the extent of the improvement observed was much higher in the SCF-EPSC (P=0.0001) and S100 (P=0.0001) liposome-treated groups, which were almost equal to the initial values of the respective groups. To compare two groups, independent sample Student’s t-tests were performed and the values (P1, P2, P3) are listed in Table 2. For the statistical differences among the four groups, one-way ANOVA (P=0.0001) showed that there were significant differences among the formulations after the treatment and the therapeutic effectiveness of the formulations (based on the STT values) was ranked by the Student–Newman–Keuls test. The order was as follows: untreated (rank 1) < Restasis®-treated (rank 2) < SCF-EPSC-liposome-treated (rank 3) < SCF-S100-liposome-treated (rank 4).

The therapeutic efficacy of liposomal CsA in producing more tears is supported by the STT report that revealed both liposomal formulations (especially SCF-S100) could be an alternative to Restasis® for the treatment of DES. The enhanced effect of liposomal formulations might be due to liposomal stabilization of the lipid layer of the tear film, which regulates and improves the process of moisturizing the eye and lid surface.37 The lipid layer in the eyes of rabbits and humans is of vastly different composition, and it is known that phospholipids might be a major constituent of the tear film lipid layer in rabbits but can destabilize the tear film lipid layer in humans.38 Thus, the effect of ophthalmic lipid supplements cannot be readily transferred from rabbits to humans. However, the other aspects of tear dynamics are similar and thus the data on CsA release on the ocular surface and the tolerability of the liposomal formulations can be expected to be relevant to the human eye. Developed liposomal formulations were much smaller and more homogeneous than Restasis®. The smaller liposomes with homogeneous-size distribution are reported to produce uniform properties of drug release, higher density of liposome packing allowed at an ocular tissue surface, and greater optical clarity in ophthalmic applications.39

Determination of the amount of CsA in rabbit tears
The concentration of CsA in rabbit tears after administering SCF-EPSC and SCF-S100 liposomal solutions and Restasis® to both eyes of rabbits is shown in Figure 3. The initial concentration of the drug in tear film was very high for all formulations because drainage and elimination processes started right after the drug was administered. The concentration of liposomal drugs observed at 10 minutes was significantly higher than that of the conventional emulsion, Restasis®. The concentrations of all formulations were lower after 30 minutes. The fast decrease of CsA concentration could be explained by the mechanical elimination of the excess of instilled volume from the cul-de-sac. Interestingly, the concentration of liposomal formulations in the tear film was higher at each time point compared to Restasis®. After 24 hours of treatment, the concentrations of CsA were found to be 285±80 ng/mL, 461±97 ng/mL, and 448±106 ng/mL for Restasis®, SCF-S100, and SCF-EPSC liposomes, respectively.

Many studies have proved that liposomes can improve corneal uptake40,41 and the cornea may act as a reservoir to release CsA.42 For ocular application, it is difficult to maintain therapeutic effects of drugs for a prolonged period of time because the liquid dosage forms have a tendency to be removed easily from the surface of the eye. The cornea may act as a reservoir in this case, and CsA is being

Figure 3 CsA concentration of tear film in rabbits (n=4).
Note: *Significant at P<0.05 versus Restasis® (Allergan Inc., Irvine, CA, USA).
Abbreviations: CsA, cyclosporin A; SCF-EPSC, supercritical fluid-mediated liposomes prepared using Lipoid EPCS; SCF-S100, supercritical fluid-mediated liposomes prepared using Lipoid S100.
released in a controlled manner. The reason for a high CsA level immediately after the instillation might be due to a burst release. Also, it could be expected that CsA would be precipitated within the limited volume of lachrymal fluid present in the precorneal environment, because of burst release, and accumulate in the cul-de-sac.\textsuperscript{42}

The pharmacokinetic parameters were calculated in order to examine the elimination of CsA from the precorneal area as shown in Table 3. Restasis\textsuperscript{8} and liposomal formulations have significantly different AUC\textsubscript{0–24 h} indicatting that the nature of the formulation affects the AUC. The highest AUC\textsubscript{0–24 h} obtained with SCF-S100 was 32.75±9.21 μg h/mg, but both liposomes had statistically similar AUC\textsubscript{0–24 h} whereas Restasis\textsuperscript{8} had a significantly lower value of 24.59±8.69 μg h/mg (\textit{P}=0.001). The data is in accordance with the CsA determination in rabbit tear film and other in vitro permeation studies. The pharmacokinetic study showed that liposomal formulations have significantly higher tear film concentration of CsA as well as AUC\textsubscript{0–24 h} compared to the marketed emulsion, Restasis\textsuperscript{8}. The release of the drug through liposomes was supposed to be prolonged, and it was observed that after 24 hours of the instillations, liposomal drugs were more predominantly present than Restasis\textsuperscript{8} in the tear film. This result suggests that the liposomal formulations might be therapeutically more effective after the ocular administration for the treatment of DES in rabbits.

### Ocular irritation tests

The Draize eye test is a governmentally endorsed method to evaluate the safety of materials meant for use in or around the eyes, by instilling ophthalmic agents onto the cornea and conjunctiva of laboratory animals.\textsuperscript{29} The Draize eye test results showed that there was no sign of discomfort appearing after the instillation of the novel SCF-S100 liposomal formulation. Grade zero was reported for all of the parameters examined. For the long-term irritation test, animals were sacrificed after the experiment and the signs of changes in the conjunctiva, cornea, and lids were macroscopically examined, and there was no difference observed between the treated and untreated groups. In conclusion, there were no signs of alteration observed after the treatment with the novel liposomal formulation.

The report of blinking in the rabbits is shown in Figure 4. There were no significant differences in the blinking of the control eyes and liposome-treated eyes (\textit{P}=0.088). On the other hand, the Restasis\textsuperscript{8}-treated group showed a significant increase in blinking after treatment with Restasis\textsuperscript{8}. In the Restasis\textsuperscript{8}-treated group, the total number of blinks observed during 2 minutes was 7.50±0.71; in control eyes, that improved significantly to 18.50±1.18 blinks (\textit{P}=0.002). The blink count of the liposome-treated eyes was significantly lower compared with the Restasis\textsuperscript{8}-treated group, and it was least in the control eyes of both groups. Ocular irritation of Restasis\textsuperscript{8} has already been reported by many groups.\textsuperscript{11,43} Restasis\textsuperscript{8} is an oil-in-water emulsion which contains castor oil that causes irritation to the eyes.

CsA at 0.05% (Restasis\textsuperscript{8}) is known to be one of the standard options for the treatment of moderate to severe DES, but because of its prominent side effects (ocular irritation) it is not patient-friendly.\textsuperscript{23} The use of corticosteroids is the other possible option. However, long-term use of corticosteroids is also limited because of numerous side effects, including ocular hypertension, cataract, and corneal thinning.\textsuperscript{24} Very interestingly, the proposed liposomal formulation showed significantly lower eye irritation compared with that of Restasis\textsuperscript{8}. A similar result was also supported by Khan et al\textsuperscript{45} where a CsA nanosphere formulation had a significantly lower irritation effect than Restasis\textsuperscript{8}. The current study showed that there was no sign of changes in the conjunctiva, cornea,
and eye lids after the acute and long-term tests. For causing less irritation of eyes, liposomal formulations are supposed to be very simple in compositions that contain naturally derived phosphatidylecholine, which is highly compatible with biological membranes, and liposomal formulations have a smaller size compared to Restasis.

**Conclusion**

To the best of our knowledge, none of the previous studies in humans or animal models have focused on the effectiveness of liposomal CsA for the treatment of DES. In the present study, the newly synthesized SCF-mediated liposomes showed significant effects such as improved tear production in dry eyes, higher tear film concentration, and less ocular irritation in the treatment of DES in rabbits. Our results strongly support that the use of liposomal formulations provide a better efficacy compared to that of Restasis; thus current formulations hold promise as an alternative therapy for DES. More specifically, SCF-mediated SCF-S100 liposomes might be an ideal candidate for further development; the cost effectiveness of the preparation was reasonable for large-scale production compared to the existing liposomal drugs. However, further investigations (eg, detailed clinical efficacy, toxicity, and other in vivo studies) will need to be carried out in humans to confirm the advantages of the newly developed liposomal formulations over the conventional product, Restasis.

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**Disclosure**

The authors report no conflicts of interest in this work. The authors alone are responsible for the content and writing of this paper.

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