Cyclosporine A Micellar Delivery System for Dry Eyes

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국 문 요 약

안구건조증을 위한 사이클로스포린 에이의 미셀 전달 시스템

본 연구의 목적은 눈에 대한 자극성이 적고 안정성이 우수한 안약을 개발하기 위한 일환으로 미셀 전달 시스템을 적용한 최적 처방을 설계하기 위한 것이다. 사이클로스포린 에이는 면역억제제로서 뿐만 아니라 건성각결막염, 쇼그렌 증후군, 안구건조증에서 눈물양을 증가시키는 효과를 나타낸다. 하지만 사이클로스포린 에이는 상대적으로 높은 분자량(1202.61)과 난용성으로 인해 안정하고 부작용이 작은 안약 제조에 어려움이 있다.

사이클로스포린 에이의 안정성과 안약으로의 특성을 개선하기 위하여 선행연구로서 다양한 계면활성제와 보조용해제에 대한 용해도 연구를 수행하였으며 그 결과 계면활성제로서 Cremophor EL, 보조용해제로서 에탄올을 사용하여 사이클로스포린 에이 미셀 전달 시스템을 단순 혼합 및 교반만으로 용이하게 제조할 수 있다는 것을 알게 되었다.

사이클로스포린 에이, 보조용해제와 첨가제들로 인한 임계미셀농도의 변화를 확인하기 위해 계면활성제 농도에 따른 입자 크기를 확인하였다. 사이클로스포린 에이의 충분한 습윤이 가능할 경우 Cremophor EL 이 1% 이상일 시에 입자크기가 약 14nm 로서 미셀이 형성된다는 것을 확인하였다. 이것은 마이크로 단위인 시판제제 사이클로스포린 에이 에멀션 제제의 입자크기보다 훨씬 작은 크기이다.

사이클로스포린 에이를 함유하는 미셀 적용 안약의 안정성을 유지할 수 있는 포뮬레이션을 확립하기 위해 pH 및 Cremophor EL, 에탄올의 농도에 따른 안정성을 상온과 4°C 에서 평가하였다. pH, 삼투압,

입자의 크기 및 함량의 변화를 60 일동안 평가하였다. 그 결과 두 온도 조건에서 모든 실험군이 안정성을 유지하였다. 조절한 pH 값에서 크게 벗어나지 않았으며 삼투압은 약 280mOsmol-320mOsmol 의 범위 안에서 ,입자 크기는 15nm-25nm 의 범위 안에서 크게 변화하지 않았다. 또한 육안으로 관찰한 외관상의 어떠한 변화도 관찰되지 않았다.

최적화된 포뮬레이션으로 미셀 적용 안약을 제조하여 점안에 의한 약력학적 데이터로 뉴질랜드 알비노 레빗에서 셔머 스트립을 이용한 눈물량 평가를 수행하였으며 눈물 분비에 관여하는 고블릿 세포를 확인하기 위해 안구 적출 후 결막을 절개하여 H&E 염색을 하여 관찰하였다. 이 때 시판되고 있는 사이클로스포린 에멀션 제제를 점안한 군과 본 연구의 미셀 적용 안약을 점안한 군을 비교하였다. 우선 아트로핀 설페이트를 5 일 동안 1 일 3 회 점안하여 안구건조증을 유발 시켰으며 사이클로스포린 에이 안약은 각각 아트로핀 설페이트 점안 5 분 뒤에 점안하였다. 그 결과 기존 시판 제제에 비교하여 미셀 적용 안약은 시간에 따른 우수한 눈물량 증가 효과를 보여주었다. 또한 기존 제제와 같이 눈물 분비에 관여하는 고블릿 세포의 회복효과도 뛰어나다는 결과를 보여주었다.

따라서 안구건조증을 위한 사이클로스포린 에이의 미셀 전달 시스템은 안정성, 치료효과 및 안약으로의 특성에 있어서 우수한 효과를 가진 기술이라 할 수 있다. 또한 물리화학적 특성 조절 및 공정효율, 스케일업 측면에서도 많은 이점을 지녔다고 할 수 있다.

핵심되는 말 : 사이클로스포린 에이, 계면활성제, 입자크기, 미셀

1. Introduction

Many different trials have been used to enhance the ocular absorption and improve the bioavailability of drugs. In the last decade, many colloidal systems (Sahoo, Dilnawaz, and Krishnakumar 2008, 144-151) and polymeric sustained delivery systems (Bourges et al. 2006, 1182-1202) have been developed for ophthalmic applications, such as nanosuspensions (Pignatello et al. 2002, 3247-3255), microemulsions (Fialho, and Da Silva-Cunha 2004, 626-632), liposomes (Shen, and Tu 2007, E371-E377), or nanocapsules (De Campos et al. 2003, 73-81). Topical administration is, obviously, the main route for the treatment of surface symtoms and diseases, such as Sjörgren syndrome or vernal keratoconjunctivitis, or more precisely, when the targeted organs are the cornea, conjunctiva, lachrymal gland or local drainage system (Lallemand et al. 2003, 307-318).

Cyclosporine A (CsA) is a cyclic undecapeptide produced by *Tolypocladium inflattum Gams and other fungi imperfecti*. This drug is now routinely used as an oral immunosuppressor for organ transplantation. It acts by selective inhibition of interleukin-2 release during the activation of T-cells and causes suppression of the cell-mediated immune response (Noble, and Markham 1995, 924-941). Ther is also reliable evidence that CsA improves tear production in dogs with keratoconjunctivitis sicca (Kaswan, Salisbury, and Ward 1989, 1210-6) and in patients with Sjörgren syndrome or dry eye sysdrome (Gündüz, and Özdemir 1994, 438-442, Laibovitz et al. 1993, 315-323). Although these effects of CsA are

believed to be the result of improvement in autoimmunological conditions in the lachrymal gland of treated subjects and animals, there is no evidence that explains the causes of the same effect on lacrimation in normal subjects and animals (Toshida, Nakayasu, and Kanai 1998, 168-73).

Fig 1. show the structure of CsA. It is known that the absolute bioavailability of cyclosporine A is the low due to the poor absorption which is related to the relatively high molecular weight, very high lipophilicity (log P = 2.92) and poor solubility in aqueous fluids (0.012 mg/mL at 25 ºC) (El Tayar et al. 1993, 3757-3764, Mondon et al. 2011, 56-65). These difficulties may be overcome through formulations aimed at improving CsA water solubility or those designed to facilitate tisuue drug penetration and remaining.

In this study, CsA micelle solutions were prepared with surfactant, cosolvent, wetting agent and viscosity thickener. Each micelle formulation investigated for the optimization of micelle solution system (pH value, osmolarity, particle size, content). And the solubility of CsA was studied. After optimization of the micelle solution system, stability of micelle solution was studied. And to evaluate effect to animals, Schirmer tear test and H & E staining were carried out in rabbit.

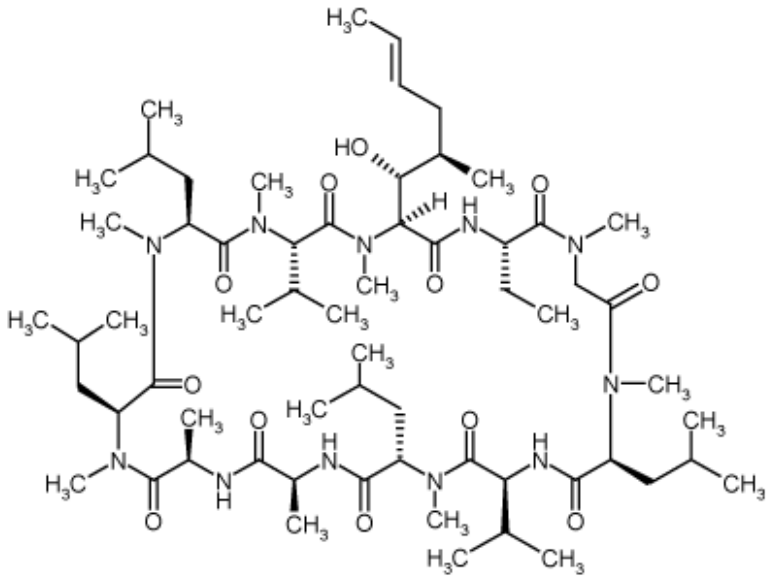

Fig 1. Chemical structure of cyclosporine A

2.Materials and method

2.1. Materials

Cyclosporine A was obtained from Concord (India). Cremophor EL (BASF, Germany), Cremophor ELP (BASF, Germany), Cremophor RH 40 (BASF, Germany), Cremophor RH 60 (BASF, Germany), Tween 80 (BASF, Germany), Span 80 (SG, NO.24784), Span 85 (Sigma Co. Ltd., USA), Labrafil M 1944 CS (Gattefosse, France), Labrafac 1349 (Gattefosse, France), Crodamol GTCC (SG, NO.666524), sodium hyaluronate (wt100,0000, Huons, Korea), glycerin (Samchun Pure Chemicals Co. Ltd, Korea), ethanol (Samchun Pure Chemicals Co. Ltd, Korea), dimethylsulfoxide (Samchun Pure Chemicals Co. Ltd, Korea), n-hexane (J.T.Baker), sodium phosphate dibasic, anhydrous (Samchun Pure Chemicals Co. Ltd, Korea), sodium phosphate, monobasic dehydrate (Samchun Pure Chemicals Co. Ltd, Korea) were used during the experiment. Atropine sulfate (Sigama Co. Ltd., USA), formalin solution (Junsei Chemical, Japan) , hematoxylin and eosin (H & E) (Sigma Aldrich, USA) were used during the experiment.

Micelle solutions were filtered through a PTFE syringe filter (Whatman International Ltd) with a pore size of 0.2 μm.

All other chemicals used were of analytical grade and double-distilled water was used.

2.2. Solubility studies of CsA in various surfactant, cosolvent

To find suitable surfactant for micelle formulation, the solubility of cyclosporine A was determined in various surfactant and cosolvent. An excess amount of CsA was added to glass vials containing 3000mg of various surfactant (Cremophor EL, Cremophor ELP, Cremophor RH40, Cremophor RH60, Tween 80, Span 80, Span 85, Labrafil M 1944 CS, Labrafac 1349, Crodamol GTCC) or 2mL of various cosolvent (ethanol, DMSO, n-hexane). All of the samples were stored for 3 days at 50 ºC with 45rpm. Then all of the samples were stored for 3 days at room temperature until the equilibrium. During the equilibrium process, samples were protected from lights. After equilibrium was achieved, each sample was filtered with 0.2 μm PTFE syringe filter (Whatman International Ltd), and the filtering sample was diluted with ethanol and the concentration of CsA was determined by HPLC.

2.3. Preparation of CsA micelles with various concentrations of surfactant and cosolvent

The cyclosporine A micelle solution was prepared according to a method described. The CsA was dissolved in surfactant with ethanol. This was solution A. The glycerin and sodium phosphate dibasic and sodium phosphate monobasic were dissolved in the aqueous phase. This was solution B. Both solutions were stirred with a magnetic stirrer separately until a homogeneous state was obtained. The homogeneous mixture was selected by visual observation. After the homogeneous solution A and B obtained, respectively, solution A and solution B were mixed. If the resulting formulation was not miscible, stirring with a magnetic stirrer process additional run. The pH was adjusted to 7.4 suitable for topical application to ocular tissue by phosphate ratio. The micelle formulation was then filtered through a PTFE filter with a pore size of 0.2 μm. A typical formulation consisted were presented in **Table 1**. Formulations of A9-A12 were not manufactured. Because CsA was not wetted enough.

Table 1. Composition of CsA micelle solution using Cremophor EL and ethanol

Weight fraction (%)					
No	Cyclosporine	PBS	Cremophor EL	Glycerin	Ethanol
A1	0.05	q.s	1	2.2	1
A2	0.05	q.s	0.5	2.2	1
A3	0.05	q.s	0.05	2.2	1
A4	0.05	q.s	0.01	2.2	1
A5	0.05	q.s	1	2.2	0.5
A6	0.05	q.s	0.5	2.2	0.5
A7	0.05	q.s	0.05	2.2	0.5
A8	0.05	q.s	0.01	2.2	0.5
A9	0.05	q.s	1	2.2	x
A10	0.05	q.s	0.5	2.2	x
A11	0.05	q.s	0.05	2.2	x
A12	0.05	q.s	0.01	2.2	x

2.4. Micelle solution characterization

The pH was examined by pH meter (S20-KS, METTLER TOLEDO). These operate at room temperature.

Osmolarity of samples were analyzed by osmometer (Vapour pressure osmometer K-7000, KNAUER, Germany). Inside temperature of osmometer was controlled to 45 ºC. 400 milliosmol / kg (12,687 g NaCl / kg) 2ml Eichlösung ampoule was used for calibration of osmometer.

2.5. Particle size analysis

The particle size and its distribution of the micelle were determined by dynamic light scattering (DLS) using electrophoretic light scattering spectrophotometer (ELS-Z, Otsuka Electronics, Japan). Each micelle solution sample was diluted to the appropriate concentration with distilled water. Measurements were carried out at 25 ºC. The particle size was expressed as the volume mean diameter (VWD) in micrometer (μm) or nanometer (nm).

2.6. Determination of total drug content

System is Agilent HPLC set consisting of a pump (Model 1260 Quat Pump VL), and auto sampler (Model 1260 ALS), and UV detector (Model 1260 VWD VL). The C18 reverse phase column (Eclipse XDB C18, 5µm, 4.6mm x 250mm, Agilent) was used at 70 ºC. The mobile phase consisted of acetonitrile:water (90:10) and was pumped at a flow rate of 1.0mL/min. The eluent was monitored at 210 nm following the injections of 20µl of CsA standard solutions in ethyl alcohol and of micelle solution samples. At specific time interval, each sample was immediately filtered through a 0.45 µm PTFE filter (Whatman International Ltd) prior analysis.

2.7. Preparation of CsA micelle composition depending on the pH or the concentration of sodium hyaluronate, Cremophor EL and ethanol

The CsA micelle solution depending on the pH or the concentration of sodium hyaluronate, Cremophor EL and ethanol were prepared according to a method of 2.3.

The CsA micelle solutions depending on the concentration of sodium hyaluronate were presented in **Table 2**. Concentrations of sodium hyaluronate were 0 %, 0.1 %, 0.3 %, 0.5 %, 0.75 % and 1%.

The CsA micelle solutions depending on the pH were adjusted pH 5.8, 6.2, 6.6, 7.0, 7.4, 7.8, and 8.2 by phosphate ratio. And residual compositions were fixed. These compositions were presented in **Table3**.

The CsA micelle solution with various concentrations of Cremophor EL and ethanol were presented in **Table 4**. Composition of D7, D8 and D9, which not contains ethanol, need stirring with a magnetic stirrer process.

The pH was adjusted to 7.4 suitable for topical application to ocular tissue by phosphate ratio. The micelle formulation was then filtered through a PTFE filter with a pore size of 0.2 μm.

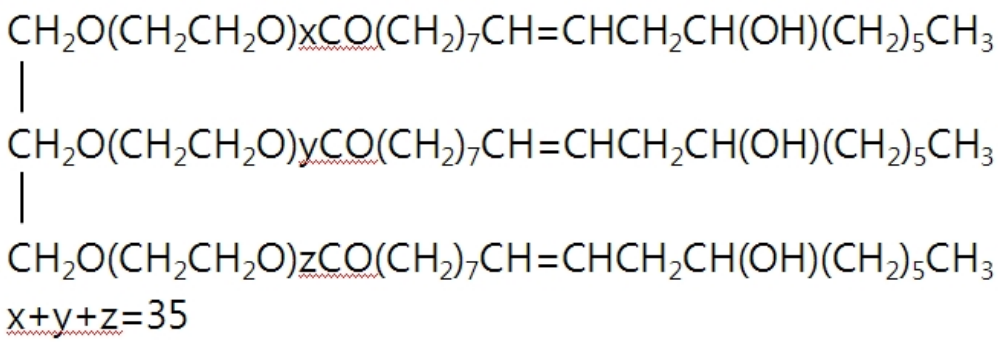

Fig 2. Chemical structure of Cremophor EL

Table 2. Composition of CsA micelle solution depending on the concentration of sod. hyaluronate

	Weight fraction (%)					
No	Cyclosporine	Water	Cremophor EL	Glycerin	Sod. hyaluronate	Ethanol
B1	0.05	q.s	5	2.2	0	1
B2	0.05	q.s	5	2.2	0.1	1
B3	0.05	q.s	5	2.2	0.3	1
B4	0.05	q.s	5	2.2	0.5	1
B5	0.05	q.s	5	2.2	0.75	1
B6	0.05	q.s	5	2.2	1	1

Table 3. Composition of CsA micelle solution depending on the pH

Weight fraction (%)						
No	Cyclosporine	PBS	Cremophor EL	Glycerin	Sod. hyaluronate	Ethanol
C1	0.05	q.s	5	2.2	0.1	1
C2	0.05	q.s	5	2.2	0.1	1
C3	0.05	q.s	5	2.2	0.1	1
C4	0.05	q.s	5	2.2	0.1	1
C5	0.05	q.s	5	2.2	0.1	1
C6	0.05	q.s	5	2.2	0.1	1
C7	0.05	q.s	5	2.2	0.1	1

Table 4. Composition of CsA micelle solution depending on the concentration of Cremophor EL and ethanol

Weight fraction (%)						
No	Cyclosporine	PBS	Cremophor EL	Glycerin	Sod. hyaluronate	Ethanol
D1	0.05	q.s	5.0	2.2	0.1	1
D2	0.05	q.s	4.0	2.2	0.1	1
D3	0.05	q.s	3.0	2.2	0.1	1
D4	0.05	q.s	5.0	2.2	0.1	0.5
D5	0.05	q.s	4.0	2.2	0.1	0.5
D6	0.05	q.s	3.0	2.2	0.1	0.5
D7	0.05	q.s	5.0	2.2	0.1	0.0
D8	0.05	q.s	4.0	2.2	0.1	0.0
D9	0.05	q.s	3.0	2.2	0.1	0.0

2.8. Long term stability assessment

The drug content, pH, osmolarity and particle size were monitored over long periods of time in the micelle formulation stored at 4 ºC and room temperature. The degree of aggregation and the phase separation were assessed visually at given time intervals. All other visible changes were recorded (Klang et al. 1999, 19-27).

2.9. Animals and treatments

Non-anaesthetised, fully awake male New Zealand albino rabbits, weighing 1.5-1.8 kg (Samtako, Korea), were used throughout. The animals were housed singly in standard cages, in a light controlled room at 19 ± 1 ºC and 50 ± 5% relative humidity, with no restriction of food or water. During the experiments the rabbits were placed in restraining boxes in a room with dim lighting. They were allowed to move their heads freely, and their eye movements were not restricted (Burgalassi et al. 1999, 229-35). The animals were divided into three groups, each group consisting at least of 8 members.

The first group received in each eye 50 µl normal saline and 50 µl atropine sulfate (AS), as the only treatment, at 9.00 a.m., 2.00 p.m. and 7.00 p.m.

Second and third groups received in the lower conjunctival sac of both eyes 50 µl of AS solution at 9.00 a.m., 2.00 p.m. and 7.00 p.m. Five minutes after each administration of AS, each group received in one eye 50 µl of the 0.9% normal

saline. The other eye received drug treatment, which is D1; cremophor EL 5 % and commercial CsA emulsion. All treatments were discontinued after 5 days.

2.10. Schirmer tear test

The Schirmer tear test was performed 1, 3, 5 days after the third administration of AS, at 9.00 p.m. The test was executed on both eyes (non-anaesthetised) of all animals, by maintaining for 2 minute a standardized sterile test strip (EagleVision, USA) into the external lower conjunctival sac. The wetted length in millimeters of the strip was taken as the test score.

2.11. H&E staining to determine the number of goblet cells

Conjunctival biopsy specimens, measuring approximately 5 x 5 mm were obtained from the same regions in the bulbar conjunctiva by surgeons following standard procedure, in control eyes and eyes of rabbit treated with the various drug formulations. Removed eye tissues from rabbit were fixed in formalin solution and embedded in paraffin. Eye tissues were then cut into 4 μm thick sections at room temperature. After deparaffining, 4 μm sections were processed and mounted on gelatin-coated slides. Then stained with hematoxylin and eosin (H&E).

Morphology was taken with bright field microscope (BX-51, Olympus) with magnification of x 40. Conjunctival epithelial morphology was evaluated, and the numbers of conjunctival epithelial goblet cells were counted along the length of three separate tissue by two independent masked observers.

As a control to study the direct effects of atropine sulfate on the ocular surface, 50 μl 1% atropine sulfate solution was applied to the ocular surface. To evaluate the curative value, 50 μl various drug formulations were applied to the ocular surface after applied atropine sulfate solution 5 minute later. Conjunctival biopsies were then performed to evaluate conjunctival goblet cell density and epithelial morphology (Dursun et al. 2002, 632-8, Kunert, Tisdale, and Gipson 2002, 330-7).

2.12. Statistical analysis

Statistical analysis was carried out using *t*-test for the tear production, and goblet cell counting. Within-group changes from baseline were analyzed by the paired *t*-test. And a $p<0.05$ was considered to be significant different.

3. Result and discussion

3.1. Solubility studies of CsA in various surfactant and cosolvent, and determine the micelle formulation

To determine the micelle formulations, CsA dissolved in various surfactant and cosolvent. The micelle solution should be clear, monophasic liquid at ambient temperature, and should have good surfactant properties to allow presentation of the drug in solution. To develop a micelle solution for ocular delivery of poorly water-soluble CsA, suitable surfactant and cosolvent need to be selected. The surfactants used in drug formulation are known to improve the bioavailability by various mechanisms. For example, the surfactants used in SMEDDS formulations are known to improve the bioavailability by various mechanisms including: (a) improved drug dissolution (Constantinides 1995, 1561-1572); (b) increased intestinal epithelial permeability (Scott Swenson, and Curatolo 1992, 39-92); (c) increased tight junction permeability (Lindmark, Nikkila, and Artursson 1995, 958-964); and (d) decreased/inhibited p-glycoprotein drug efflux (Lo, Hsu, and Huang 1998, 3005-3009, Nerurkar, Burton, and Borchardt 1996, 528-534, Yu et al. 1999, 1812-1817).

In this surfactant and cosolvent studies, the solubility of CsA in ten surfactants (Cremophor EL, Cremophor ELP, Cremophor RH 40, Cremophor RH 60, Tween 80, Span 80, Span 85, Labrafil M 1944 CS, Labrafac 1349, Crodamol GTCC) and

cosolvent (ethanol, DMSO, n-hexane) were measured. The solubility of CsA in various surfactants and cosolvents were presented in **Table 5.**

Typically, nonionic surfactants were the major type of surface active agents used in ophthalmic delivery systems since their advantages with respect to compatibility, stability, and toxicity are quite significant compared to cationic, anionic or amphoteric counterparts. They are generally less toxic, less hemolytic, and less irritating to the ocular surface, and tend to maintain near physiological pH values when in solution (Jiao 2008, 1663-1673). Especially Tween 80 and Cremophor EL as nonionic surfactants were widely applied to pharmaceutical preparations including ophthalmic preparations (Radomska-Soukharev, and Wojciechowska 2005, 465-71). Generally Tween 80 was classified as practically nonirritant and Cremophor EL caused only slight reddening of the rabbit conjunctiva, and this disappeared within a few hours (Ammar et al. 2009, 808-819). In other words, Tween 80 and Cremophor EL were few irritate in ocular delivery system. And the lower core polarity the higher effectiveness of the micelle solubilization in nonionic surfactants. Micelle core polarity value of Tween 80 and Cremophor EL were lower than other nonionic surfactants, respectively, 1.13 and 1.05 (Croy, and Kwon 2005, 2345-2354).

Two nonionic surfactants were also used for CsA formulation. The Gengraf (Abbot) cyclosporine A formulation is a hard gelatin capsule containing 25 or 50 mg of cyclosporine A dissolved in ethanol, polyoxyl 35 castor oil (Cremophor EL) and polysorbate 80 (Tween 80) and forms an aqueous dispersion in an aqueous

environment. The Sandimmune (Novartis) cyclosporine A injectable formulation contain just cremophor EL and ethyl alcohol (Strickley 2004, 201-230).

In this study, solubility of CsA with Tween 80 was lower than solubility of CsA with Cremophor EL. Solubility of CsA with Cremophor EL was 43.61 ± 2.88 (mg/g) and solubility of CsA with Cremophor ELP, purified Cremophor EL, was 49.78 ± 3.21 (mg/g). Solubility of CsA with Tween 80 was just 27.49 ± 1.48 (mg/g). This is a significant difference between two surfactants.

Although solubility of Span 80 (50.30 ± 5.81 (mg/g)) and that of Span 85 (46.37 ± 8.20 (mg/g)) were almost similar to solubility of Cremophor EL, they generally not used in ocular delivery system, and they not have regulation of FDA ophthalmic inactive ingredient. And based on our experience, formulation contained Labrafil were irritant.

So, we choose Cremophor EL for surfactant in our drug formulations. Maximum potency of Cremophor EL (polyoxyl 35 castor oil) as inactive ingredient for ophthalmic solution dosage form was 5%. But Cremophor EL increased the permeability of rabbit corneas to CsA (bijl 2002). Due to these factors, we applied concentration of Cremophor EL were 3%, 4% and 5%.

And in case of cosolvent, ethanol was typically used for ocular delivery system. And solubility of CsA with ethanol was higher than that of others, as 518.38 ± 13.06. Solubility of CsA with DMSO and n-hexane were 254.97 ± 3.16 and 3.05 ± 0.01, respectively.

Cyclosporine A 0.05 % and 0.1 % were deemed the most appropriate formulations for future clinical studies because no additional benefits were observed with the higher concentrations. And cyclosporine A 0.05 % produced the most consistent improvements in patient symptoms (such as sandy/gritty feeling and ocular dryness) (Stevenson, Tauber, and Reis 2000, 967-74).

Glycerin was applied for wetting agent and tonicity adjusting agent (Denick 1998). According to FDA inactive ingredient, the maximum potency of glycerin ophthalmic; emulsion is 2.2%. This concentration of glycerin was generally employed for ophthalmic administration like nanosphere (Khan et al. 2012, 275-276) and microemulsion (Gan et al. 2009, 143-149).

The polyanionic substance frequently proposed as a viscosity thickener in ophthalmic formulations is hyaluronic acid (Cantoro 1988). And hyaluronic acid was detected to excellent muco-adhesive properties (Saettone et al. 1989, 203-212).

Based on these ground, we prepared drug formulation and conducted experiments.

Table 5. Solubility of CsA in various surfactants and cosolvent (n=3, mean ± S.D.)

Excipients		Solubility	(mg/g)
Surfactants	Cremophor EL	43.61	±2.88
	Cremophor ELP	49.78	±3.21
	Cremophor RH 40	38.96	±1.54
	Cremophor RH 60	29.33	±0.28
	Tween 80	27.49	±1.48
	Span 80	50.30	±5.81
	Span 85	46.37	±8.20
	Labrafil M 1944 CS	48.79	±3.41
	Labrafac 1349	36.67	±1.71
	Crodamol GTCC	15.66	±1.68
Cosolvent	ethanol	518.38	±13.06
	DMSO	254.97	±3.16
	n-hexane	3.05	±0.01

3.2. Micelle solution characterization

Normal tears have a pH of about 7.4 and possess some buffer capacity (U.S Pharmacopeia). We also adjusted pH 7.4 and used phosphate buffer for aqueous background.

Tear osmolarity correlated significantly with dry eye severity grade. Ramification of dry eye disease that involves progressively elevated tear osmolarity with worsening disease severity. Although it is important to keep in mind that tear osmolarity cannot be used as the sole indicator of dry eye disease, its positive correlation with dry eye severity grade lends support to considering the use of tear osmolarity as a biomarker for dry eye disease severity (Suzuki et al. 2010, 4557-61). So, it is important to control the osmolarity of drug formulations. Lachrymal fluid is isotonic with blood, having an isotonic value corresponding to that of a 0.9 % sodium chloride solution (about 300 mOsmol). But the eye can tolerated isotonicity values as low as that of a 0.6 % sodium chloride solution and as high as that of a 2.0 % sodium chloride solution without marked discomport (U.S Pharmacopeia). We adjusted osmolarity in this range.

As shown in **Table 7.**, pH value was grew higher with sod. hyaluronate content grows. pH value of B1, concentration of sod. hyaluronate was zero, was 4.18 ± 0.015. And pH value of B6, concentration of sod. hyaluronate was1 %, was 5.12 ± 0.050.

pH adjusted to various value, in **Table 8.**, were practically precise.

The pH which were adjusted to 7.4 by phosphate ratio, were almost constant in **Table 6.** and **Table 9**.

All of the osmolarity were within the range.

Table 6. pH and osmolarity of the drug formulation of A1-A12 (mean ± S.D., n=3)

Composition (%)						pH	Osmolarity (mOsmol)
No	CsA	Cremophor EL	Glycerin	Ethanol	PBS		
A1	0.05	1	2.2	1	q.s	7.35±0.035	356.1±12.66
A2	0.05	0.5	2.2	1	q.s	7.31±0.021	359.33± 17.09
A3	0.05	0.05	2.2	1	q.s	7.32±0.035	377.77±15.20
A4	0.05	0.01	2.2	1	q.s	7.32±0.032	369.67±12.38
A5	0.05	1	2.2	0.5	q.s	7.28±0.026	351.13±4.50
A6	0.05	0.5	2.2	0.5	q.s	7.27±0.021	360.87±16.42
A7	0.05	0.05	2.2	0.5	q.s	7.30±0.032	391.90±10.39
A8	0.05	0.01	2.2	0.5	q.s	7.28±0.015	379.50±14.78
A9	0.05	1	2.2	x	q.s	-	-
A10	0.05	0.5	2.2	x	q.s	-	-
A11	0.05	0.05	2.2	x	q.s	-	-
A12	0.05	0.01	2.2	x	q.s	-	-

Table 7. pH and osmolarity of the drug formulation of B1-B6 (mean ± S.D., n=3)

Composition (%)							pH	Osmolarity (mOsmol)
No	CsA	Cremophor EL	Glycerin	Sod. hyaluronate	Ethanol	Water		
B1	0.05	5	2.2	0	1	q.s	4.18±0.015	310.53±7.44
B2	0.05	5	2.2	0.1	1	q.s	4.43±0.045	300.00±6.85
B3	0.05	5	2.2	0.3	1	q.s	4.67±0.042	302.5±6.94
B4	0.05	5	2.2	0.5	1	q.s	4.83±0.025	303.83±5.35
B5	0.05	5	2.2	0.75	1	q.s	5.02±0.026	309.63±8.38
B6	0.05	5	2.2	1	1	q.s	5.12±0.050	297.03±7.92

Table 8. pH and osmolarity of the drug formulation of C1-C7 (mean ± S.D., n=3)

Composition (%)							pH	Osmolarity (mOsmol)
No	CsA	Cremophor EL	Glycerin	Sod. hyaluronate	Ethanol	PBS		
C1	0.05	5	2.2	0.1	1	q.s	5.75±0.010	306.53± 10.22
C2	0.05	5	2.2	0.1	1	q.s	6.19±0.012	307.80±1.15
C3	0.05	5	2.2	0.1	1	q.s	6.60±0.015	311.27±4.81
C4	0.05	5	2.2	0.1	1	q.s	7.02±0.015	300.00± 12.13
C5	0.05	5	2.2	0.1	1	q.s	7.40±0.015	301.73±6.63
C6	0.05	5	2.2	0.1	1	q.s	7.82±0.012	304.40±4.78
C7	0.05	5	2.2	0.1	1	q.s	8.22±0.006	304.07±2.99

Table 9. pH and osmolarity of the drug formulation of D1-D9 (mean ± S.D., n=3)

Composition (%)							pH	Osmolarity (mOsmol)
No	CsA	Cremophor EL	Glycerin	Sod. hyaluronate	Ethanol	PBS		
D1	0.05	5	2.2	0.1	1	q.s	7.45±0.031	306.37±3.46
D2	0.05	4	2.2	0.1	1	q.s	7.46±0.006	311.13±4.30
D3	0.05	3	2.2	0.1	1	q.s	7.42±0.031	309.53± 12.11
D4	0.05	5	2.2	0.1	0.5	q.s	7.44±0.006	299.63±2.68
D5	0.05	4	2.2	0.1	0.5	q.s	7.47±0.021	298.30±9.54
D6	0.05	3	2.2	0.1	0.5	q.s	7.43±0.025	305.50±3.51
D7	0.05	5	2.2	0.1	x	q.s	7.43±0.025	311.00± 10.65
D8	0.05	4	2.2	0.1	x	q.s	7.42±0.021	316.77± 12.86
D9	0.05	3	2.2	0.1	x	q.s	7.41±0.010	317.40±7.64

3.3. Micelle solution droplet size analysis

The smaller droplet size in ophthalmic delivery system improved reducing ocular irritation. In this study, the particle size of 1 phase micelle formulation presented in **Table 10** – **Table13**.

As show in **Table 10.**, droplet size of A1 and A5 were 14.53 ± 0.40 and 14.73 ± 0.21. And other formulation were relatively larger than A1, A5. The reason of these are critical micelle concentration (CMC) of Cremophor EL. Concentration of Cremophor EL in A1 and A5 was 1 %. This concentration above CMC of Cremophor EL, 0.02 % (wt%) (Jiao 2008, 1663-1673). Although concentration of Cremophor EL above the CMC, we considered peptide drug and surfactant interactions. (van Tellingen et al. 1999, 330-5). It is well known that addition of solvents, which act as water structure breakers decrease the hydrophobic effect resulting into an increase in the CMC of ionic surfactants (Sujit Kumar Shah 2012, 37-45). So we choose that concentration of Cremophor EL were more than 1 %.

As shown in **Table 11.**, droplet size of micelles were grew higher with sod. hyaluronate content grows. Droplet size of B1, concentration of sod. hyaluronate was zero, was 15.87 ± 0.61 nm. And droplet size of B6, concentration of sod. hyaluronate was1 %, was 34.00 ± 1.14 nm.

Formed micelle formulation were almost same, **Table 12.** and **Table 13.**

Table 10. Droplet size of the drug formulation of A1-A12 (mean ± S.D., n=3)

Composition (%)						Mean particle size (nm)
No	CsA	Cremophor EL	Glycerin	Ethanol	PBS	
A1	0.05	1	2.2	1	q.s	14.53±0.40
A2	0.05	0.5	2.2	1	q.s	245.17±4.31
A3	0.05	0.05	2.2	1	q.s	168.10±2.40
A4	0.05	0.01	2.2	1	q.s	199.77±7.23
A5	0.05	1	2.2	0.5	q.s	14.73±0.21
A6	0.05	0.5	2.2	0.5	q.s	213.93±6.97
A7	0.05	0.05	2.2	0.5	q.s	192.13±3.76
A8	0.05	0.01	2.2	0.5	q.s	359.43±6.10
A9	0.05	1	2.2	x	q.s	-
A10	0.05	0.5	2.2	x	q.s	-
A11	0.05	0.05	2.2	x	q.s	-
A12	0.05	0.01	2.2	x	q.s	-

Table 11. Droplet size of the drug formulation of B1-B6 (mean ± S.D., n=3)

	Composition (%)						Mean particle size (nm)
No	CsA	Cremophor EL	Glycerin	Sod. hyaluronate	Ethanol	Water	
B1	0.05	5	2.2	0	1	q.s	15.87±0.61
B2	0.05	5	2.2	0.1	1	q.s	17.70±0.96
B3	0.05	5	2.2	0.3	1	q.s	22.27±1.46
B4	0.05	5	2.2	0.5	1	q.s	24.70±1.91
B5	0.05	5	2.2	0.75	1	q.s	30.20±0.95
B6	0.05	5	2.2	1	1	q.s	34.00±1.14

Table 12. Droplet size of the drug formulation of C1-C7 (mean ± S.D., n=3)

	Composition (%)						Mean particle size (nm)
No	CsA	Cremophor EL	Glycerin	Sod. hyaluronate	Ethanol	PBS	
C1	0.05	5	2.2	0.1	1	q.s	20.47±1.00
C2	0.05	5	2.2	0.1	1	q.s	21.43±1.22
C3	0.05	5	2.2	0.1	1	q.s	20.50±1.00
C4	0.05	5	2.2	0.1	1	q.s	20.57±2.05
C5	0.05	5	2.2	0.1	1	q.s	20.47±3.00
C6	0.05	5	2.2	0.1	1	q.s	19.67±0.76
C7	0.05	5	2.2	0.1	1	q.s	20.60±0.70

Table 13. Droplet size of the drug formulation of D1-D9 (mean ± S.D., n=3)

	Composition (%)						Mean particle size (nm)
No	CsA	Cremophor EL	Glycerin	Sod. hyaluronate	Ethanol	PBS	
D1	0.05	5	2.2	0.1	1	q.s	17.20±0.30
D2	0.05	4	2.2	0.1	1	q.s	17.53±0.06
D3	0.05	3	2.2	0.1	1	q.s	17.37±0.25
D4	0.05	5	2.2	0.1	0.5	q.s	17.33±0.29
D5	0.05	4	2.2	0.1	0.5	q.s	17.53±0.38
D6	0.05	3	2.2	0.1	0.5	q.s	17.30±0.10
D7	0.05	5	2.2	0.1	x	q.s	17.27±0.31
D8	0.05	4	2.2	0.1	x	q.s	17.73±0.15
D9	0.05	3	2.2	0.1	x	q.s	17.50±0.26

3.4. Long term stability assessment

C1-C7 formulation and D1-D9 formulation were selected for determination of long-term stability. All of the micelle samples were divided and each samples were stored at 4 ºC and room temperature respectively. And samples were measured pH value, osmolarity, droplet size and content. The results of formulations C1-C7 were presented in **Table14.**, **Table 16.**, **Table 18.** and **Figure 3.**, **Figure 5., Figure7.**, **Figure 9.** stored at room temperature. And **Table15.**, **Table 17.**, **Table 19.** and **Figure 4.**, **Figure 6., Figure8.**, **Figure10.** were stored at 4 ºC. The results of formulations D1-D9 were presented in **Table 20.**, **Table 22.**, **Table 24. a**nd **Figure 11.**, **Figure 13.**, **Figure 15.**, **Figure 17.** stored at room temperature. And **Table 21.**, **Table 23.**, **Table 25. a**nd **Figure 12.**, **Figure 14.**, **Figure 16.**, **Figure 18.** were stored at 4 ºC.

The significant change of pH, osmolarity, droplet size and content measurement are not shown. And micelle formulations were also stable physically following storage at both temperature. In other words, visible changes were not observed.

Table 14. pH value of C1-C7 before and after storage at room temperature (mean ± S.D., n=3)

No	Control pH	pH							
		Initial	After 4 day	After 7 day	After 10 day	After 14 day	After 21 day	After 1 month	After 2 month
C1	5.8	5.75±0.010	5.78±0.076	5.78±0.083	5.81±0.057	5.78±0.080	5.81±0.053	5.83±0.093	5.79±0.047
C2	6.2	6.19±0.012	6.16±0.061	6.21±0.178	6.19±0.035	6.20±0.096	6.22±0.060	6.23±0.078	6.20±0.070
C3	6.6	6.60±0.015	6.62±0.091	6.62±0.085	6.64±0.055	6.62±0.106	6.57±0.127	6.59±0.068	6.60±0.061
C4	7.0	7.02±0.015	7.06±0.125	7.03±0.075	7.04±0.032	7.05±0.045	7.04±0.137	7.05±0.087	6.99±0.021
C5	7.4	7.40±0.015	7.41±0.071	7.42±0.115	7.45±0.046	7.37±0.120	7.39±0.081	7.43±0.092	7.36±0.062
C6	7.8	7.82±0.012	7.79±0.051	7.79±0.092	7.84±0.035	7.78±0.105	7.77±0.082	7.82±0.082	7.79±0.025
C7	8.2	8.22±0.006	8.24±0.103	8.19±0.095	8.25±0.047	8.22±0.112	8.17±0.103	8.19±0.114	8.18±0.006

Table 15. pH value of C1-C7 before and after storage at 4 ºC (mean ± S.D., n=3)

No	Control pH	pH							
		Initial	After 4 day	After 7 day	After 10 day	After 14 day	After 21 day	After 1 month	After 2 month
C1	5.8	5.75±0.010	5.81±0.020	5.80±0.040	5.80±0.101	5.79±0.097	5.79±0.067	5.77±0.187	5.84±0.035
C2	6.2	6.19±0.012	6.27±0.042	6.25±0.030	6.18±0.092	6.22±0.110	6.21±0.061	6.24±0.171	6.25±0.091
C3	6.6	6.60±0.015	6.67±0.075	6.70±0.090	6.60±0.101	6.61±0.123	6.61±0.055	6.60±0.132	6.62±0.108
C4	7.0	7.02±0.015	7.04±0.030	7.09±0.061	7.03±0.165	7.04±0.160	7.05±0.050	7.00±0.156	7.03±0.165
C5	7.4	7.40±0.015	7.46±0.057	7.47±0.046	7.43±0.080	7.37±0.116	7.46±0.098	7.40±0.131	7.35±0.050
C6	7.8	7.82±0.012	7.83±0.061	7.83±0.061	7.79±0.055	7.76±0.140	7.76±0.026	7.77±0.108	7.74±0.134
C7	8.2	8.22±0.006	8.29±0.072	8.28±0.075	8.18±0.071	8.19±0.032	8.18±0.091	8.18±0.170	8.17±0.155

Table 16. Osmolarity of C1-C7 before and after storage at room temperature (mean ± S.D., n=3)

No	Control pH	Osmolarity (mOsmol)							
		Initial	After 4 day	After 7 day	After 10 day	After 14 day	After 21 day	After 1 month	After 2 month
C1	5.8	312.17±12.25	301.67±15.93	309.73±10.23	298.50±7.86	292.47±5.61	296.27±18.41	305.57±3.79	294.73±6.31
C2	6.2	307.80±1.15	301.20±5.48	296.20±8.20	303.60±20.81	301.90±4.88	302.93±4.14	311.97±3.17	292.97±5.12
C3	6.6	311.27±4.81	305.63±21.72	315.37±1.79	316.90±5.77	294.57±6.51	303.30±5.01	311.03±5.13	301.10±6.40
C4	7.0	300.00±12.13	300.97±5.67	297.40±9.97	290.67±6.53	295.27±7.74	304.93±3.07	309.37±8.46	300.30±2.14
C5	7.4	301.73±6.63	300.97±11.66	297.20±6.36	295.83±1.46	299.50±10.84	313.23±3.15	300.37±5.42	299.40±10.81
C6	7.8	304.40±4.78	305.27±6.86	298.10±3.14	296.80±6.77	299.47±14.48	299.13±16.23	307.00±19.35	305.57±9.05
C7	8.2	304.07±2.99	298.57±9.53	298.37±1.92	294.23±12.15	300.53±0.74	300.57±7.41	305.93±6.86	303.23±4.69

Table 17. Osmolarity of C1-C7 before and after storage at 4 ºC (mean ± S.D., n=3)

No	Control pH	Osmolarity (mOsmol)							
		Initial	After 4 day	After 7 day	After 10 day	After 14 day	After 21 day	After 1 month	After 2 month
C1	5.8	312.17±12.25	318.57±23.18	308.97±12.74	299.53±6.78	296.96±8.41	309.63±9.58	292.07±4.45	284.60±12.77
C2	6.2	307.80±1.15	321.77±18.57	303.10±6.91	297.80±8.54	309.67±8.75	304.57±8.10	308.60±1.10	298.27±4.20
C3	6.6	311.27±4.81	324.10±19.47	306.90±10.94	301.20±6.62	304.83±9.17	309.80±17.02	311.23±11.19	299.83±2.14
C4	7.0	300.00±12.13	314.43±3.52	297.01±3.98	300.50±3.52	297.27±8.39	305.17±6.81	310.63±9.84	300.93±6.11
C5	7.4	301.73±6.63	314.77±6.67	299.33±10.49	294.23±12.61	299.93±4.22	306.40±4.53	304.07±4.50	297.73±10.25
C6	7.8	304.40±4.78	310.30±5.91	308.63±18.23	301.37±7.66	300.23±15.41	306.10±0.95	300.50±8.05	299.13±3.83
C7	8.2	304.07±2.99	303.47±13.88	295.97±5.46	302.10±14.81	304.03±5.50	303.87±2.89	311.17±3.54	300.30±5.29

Table 18. Mean particle size of C1-C7 before and after storage at room temperature (mean ± S.D., n=3)

No	Control pH	Mean particle size (nm)							
		Initial	After 4 day	After 7 day	After 10 day	After 14 day	After 21 day	After 1 month	After 2 month
C1	5.8	20.47±1.00	20.77±1.02	20.60±0.85	18.17±1.59	19.10±1.41	18.60±2.00	21.53±2.05	18.50±0.56
C2	6.2	21.43±1.22	19.23±1.52	20.83±1.15	18.73±0.71	19.13±1.00	18.43±1.55	20.60±1.85	19.77±0.21
C3	6.6	20.50±1.00	20.57±1.79	22.63±1.95	19.20±1.93	19.30±1.32	18.93±1.33	20.53±1.00	19.50±1.15
C4	7.0	20.57±2.05	2067±1.10	21.67±1.66	19.50±2.11	19.03±1.36	19.07±2.18	18.80±0.85	17.87±0.58
C5	7.4	20.47±3.00	20.97±2.15	21.30±1.04	19.77±2.42	18.97±1.07	18.73±1.63	19.03±0.51	18.70±0.44
C6	7.8	19.67±0.76	20.93±1.25	21.63±0.23	19.03±2.25	18.83±0.68	19.73±1.17	19.40±0.75	18.23±0.57
C7	8.2	20.60±0.70	21.30±0.70	21.57±1.37	18.83±1.48	19.13±1.19	18.50±1.40	18.77±1.56	17.77±0.35

Table 19. Mean particle size of C1-C7 before and after storage at 4 ºC (mean ± S.D., n=3)

No	Control pH	Mean particle size (nm)							
		Initial	After 4 day	After 7 day	After 10 day	After 14 day	After 21 day	After 1 month	After 2 month
C1	5.8	20.47±1.00	20.47±0.95	21.00±1.31	20.10±2.31	24.53±1.05	20.73±1.96	20.23±0.15	16.70±0.60
C2	6.2	21.43±1.22	19.97±1.46	21.50±1.95	20.07±1.27	23.27±0.95	20.40±1.08	20.60±2.26	16.90±0.46
C3	6.6	20.50±1.00	19.33±2.08	20.20±2.34	19.80±1.47	19.77±0.15	20.07±1.45	21.50±1.47	17.30±0.75
C4	7.0	20.57±2.05	19.87±1.42	20.60±1.01	19.97±2.21	19.73±0.32	20.20±2.86	20.10±2.29	16.93±0.47
C5	7.4	20.47±3.00	18.63±1.63	20.27±2.04	19.63±1.80	20.50±0.61	20.40±2.50	20.27±1.37	17.00±0.46
C6	7.8	19.67±0.76	20.07±2.30	21.83±1.86	20.00±2.35	20.33±0.84	19.27±1.70	21.20±2.46	16.97±0.15
C7	8.2	20.60±0.70	19.70±1.59	21.73±0.90	19.80±2.21	20.13±1.23	18.63±1.33	19.77±1.31	17.33±0.55

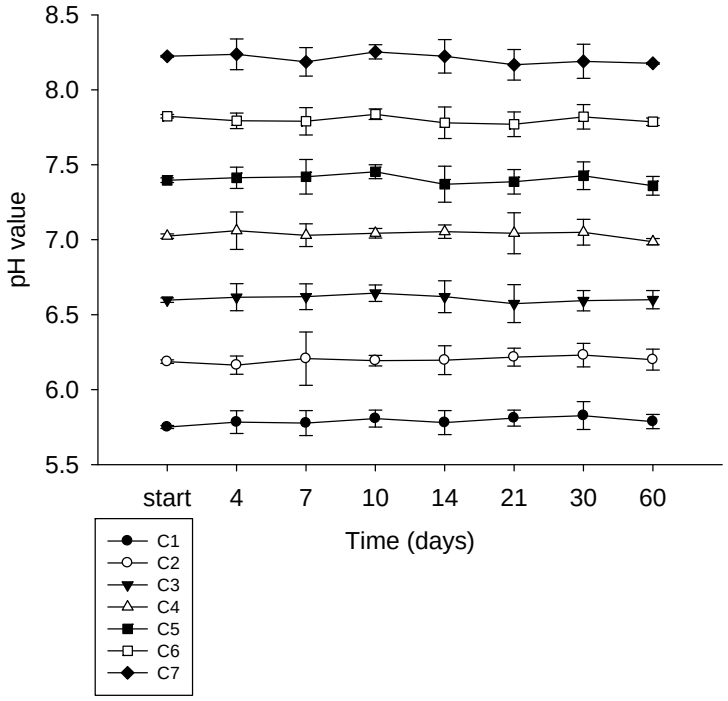

Fig 3. pH profile of C1-C7 stored at room temperature

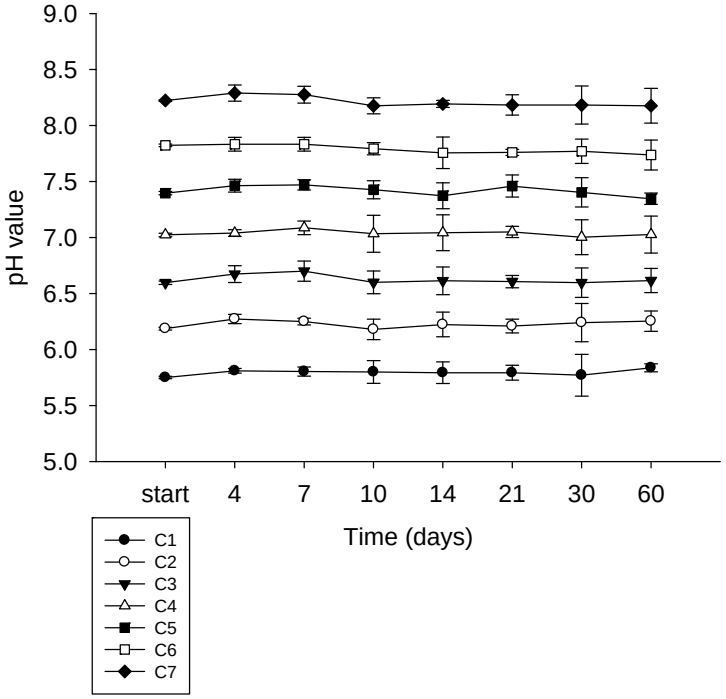

Fig 4. pH profile of C1-C7 stored at 4 ºC

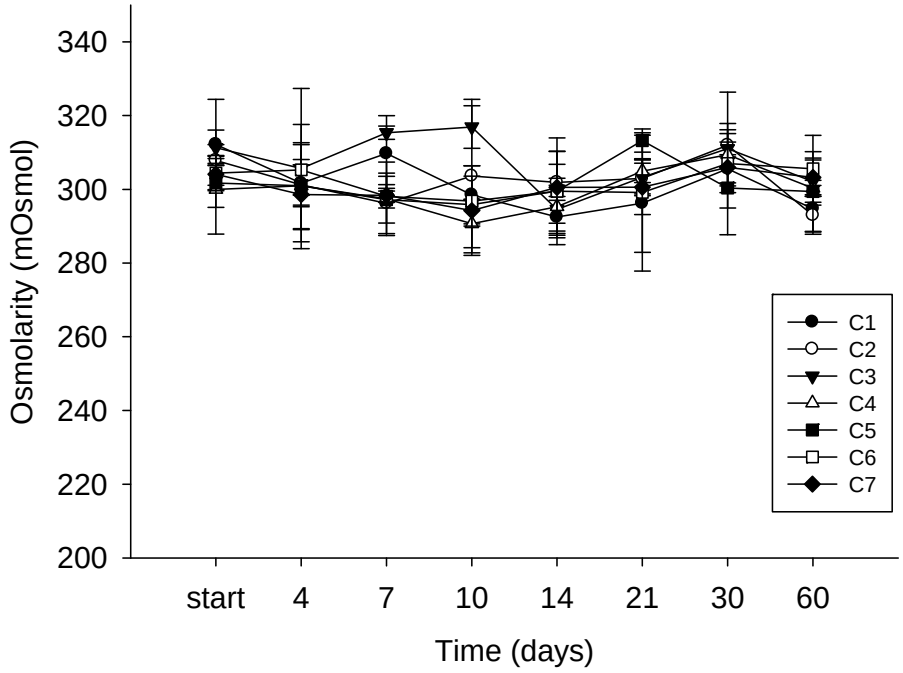

Fig 5. Osmolarity profile of C1-C7 stored at room temperature

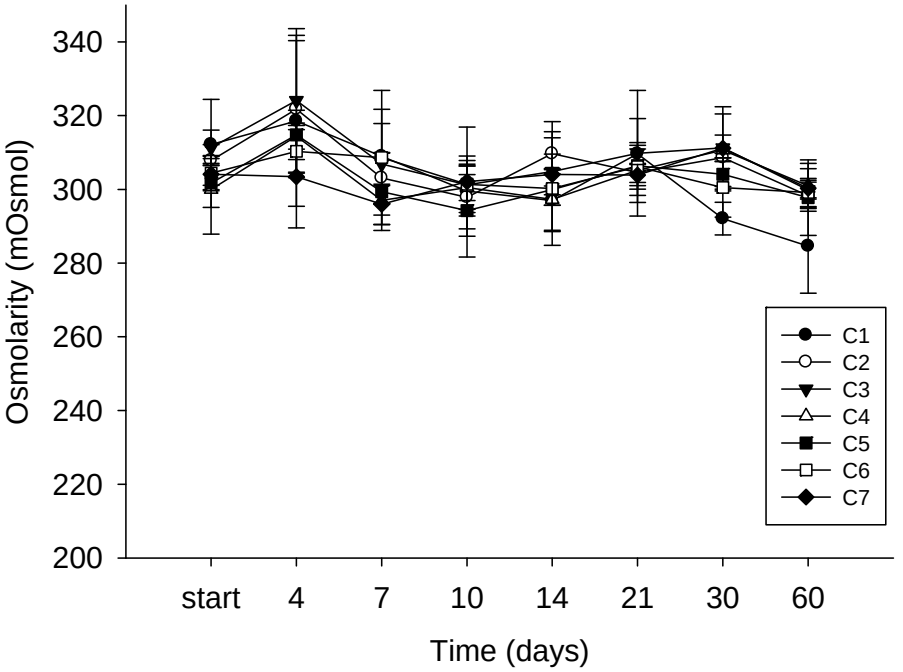

Fig 6. Osmolarity profile of C1-C7 stored at 4ºC

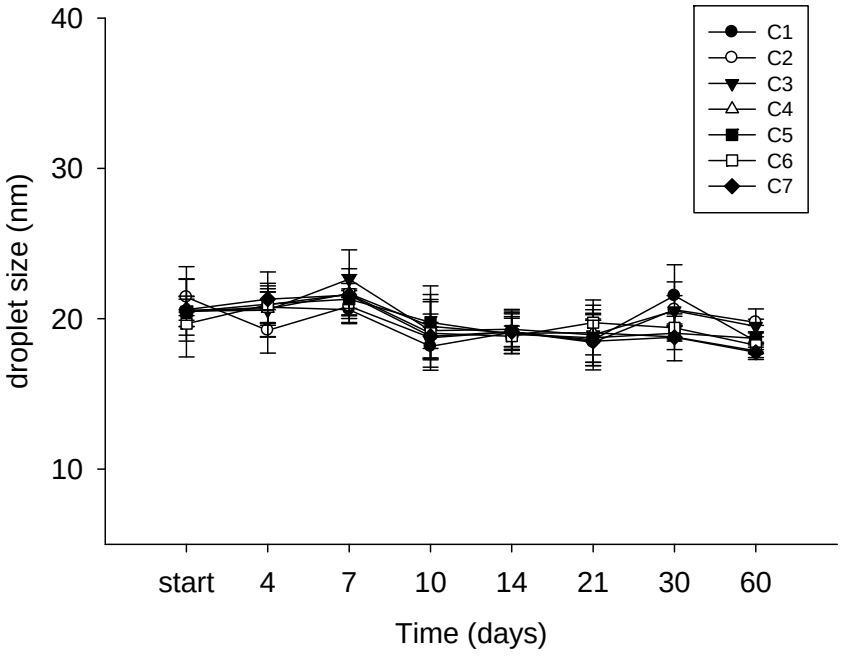

Fig 7. Droplet size profile of C1-C7 stored at room temperature

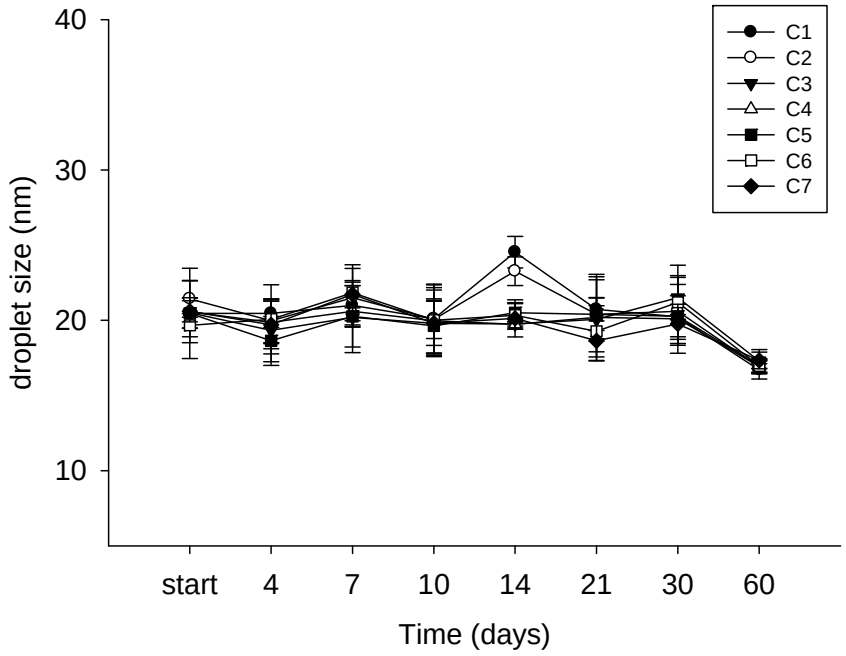

Fig 8. Droplet size profile of C1-C7 stored at 4ºC

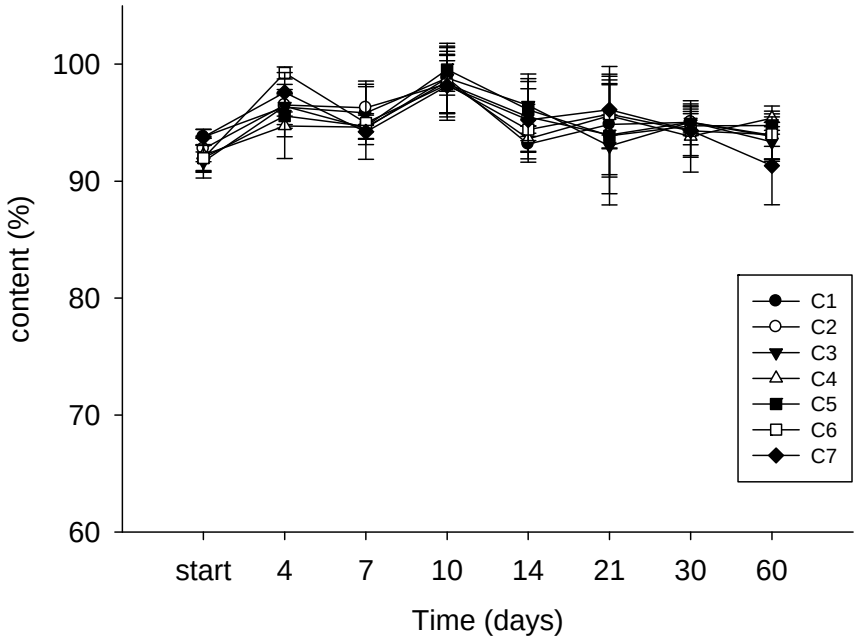

Fig 9. Content profile of C1-C7 stored at room temperature

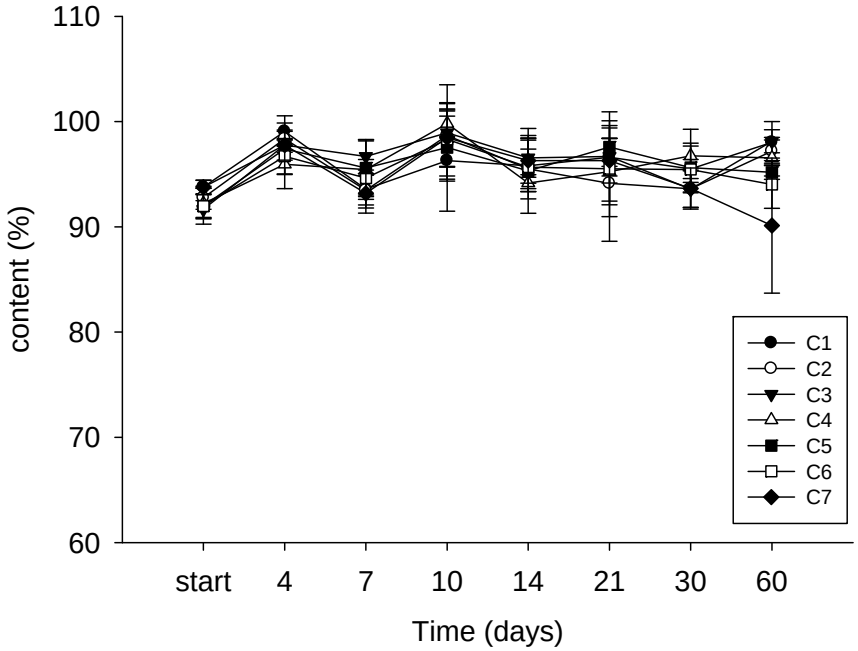

Fig 10. Content profile of C1-C7 stored at 4ºC

Table 20. pH value of D1-D9 before and after storage at room temperature (mean ± S.D., n=3)

No	Ratio	pH							
		Initial	After 4 day	After 7 day	After 10 day	After 14 day	After 21 day	After 1 month	After 2 month
D1	5:1	7.45±0.031	7.44±0.032	7.38±0.085	7.44±0.023	7.43±0.067	7.43±0.044	7.41±0.091	7.39±0.067
D2	4:1	7.46±0.006	7.42±0.070	7.36±0.095	7.42±0.030	7.40±0.015	7.44±0.035	7.41±0.095	7.40±0.046
D3	3:1	7.42±0.031	7.41±0.032	7.38±0.081	7.40±0.050	7.42±0.055	7.46±0.044	7.47±0.096	7.42±0.053
D4	5:0.5	7.44±0.006	7.44±0.050	7.39±0.072	7.38±0.067	7.43±0.049	7.40±0.023	7.44±0.061	7.40±0.012
D5	4:0.5	7.47±0.021	7.38±0.061	7.38±0.096	7.38±0.083	7.42±0.044	7.44±0.067	7.41±0.106	7.41±0.020
D6	3:0.5	7.43±0.025	7.42±0.010	7.41±0.046	7.39±0.067	7.43±0.053	7.42±0.060	7.41±0.067	7.41±0.051
D7	5:0	7.43±0.025	7.36±0.047	7.38±0.058	7.40±0.021	7.42±0.064	7.40±0.023	7.41±0.059	7.40±0.026
D8	4:0	7.42±0.021	7.37±0.070	7.36±0.070	7.39±0.044	7.39±0.055	7.42±0.031	7.38±0.049	7.38±0.059
D9	3:0	7.41±0.010	7.39±0.101	7.35±0.071	7.42±0.070	7.39±0.035	7.41±0.055	7.42±0.049	7.39±0.015

Table 21. pH value of D1-D9 before and after storage at 4 ºC (mean ± S.D., n=3)

No	Ratio	pH							
		Initial	After 4 day	After 7 day	After 10 day	After 14 day	After 21 day	After 1 month	After 2 month
D1	5:1	7.45±0.031	7.37±0.067	7.42±0.093	7.40±0.060	7.39±0.040	7.42±0.095	7.38±0.087	7.42±0.031
D2	4:1	7.46±0.006	7.38±0.076	7.42±0.042	7.40±0.078	7.36±0.087	7.42±0.056	7.42±0.040	7.39±0.072
D3	3:1	7.42±0.031	7.41±0.021	7.43±0.104	7.42±0.030	7.40±0.032	7.40±0.082	7.38±0.098	7.39±0.061
D4	5:0.5	7.44±0.006	7.40±0.029	7.46±0.045	7.42±0.026	7.36±0.062	7.43±0.093	7.38±0.084	7.41±0.044
D5	4:0.5	7.47±0.021	7.40±0.091	7.43±0.046	7.44±0.023	7.36±0.062	7.43±0.050	7.40±0.025	7.37±0.053
D6	3:0.5	7.43±0.025	7.43±0.057	7.41±0.040	7.39±0.076	7.37±0.060	7.41±0.070	7.39±0.055	7.41±0.075
D7	5:0	7.43±0.025	7.37±0.053	7.39±0.068	7.42±0.031	7.38±0.026	7.44±0.047	7.39±0.065	7.39±0.086
D8	4:0	7.42±0.021	7.40±0.015	7.44±0.045	7.39±0.050	7.39±0.046	7.44±0.057	7.37±0.086	7.41±0.021
D9	3:0	7.41±0.010	7.39±0.090	7.40±0.015	7.39±0.045	7.36±0.056	7.42±0.025	7.40±0.038	7.40±0.010

Table 22. Osmolarity of D1-D9 before and after storage at room temperature (mean ± S.D., n=3)

No	Ratio	Osmolarity (mOsmol)							
		Initial	After 4 day	After 7 day	After 10 day	After 14 day	After 21 day	After 1 month	After 2 month
D1	5:1	306.37±3.46	305.03±9.34	302.87±6.67	312.70±7.69	303.93±1.91	303.57±6.02	301.80±0.66	300.30±3.02
D2	4:1	311.13±4.30	302.50±6.00	301.97±0.40	313.53±10.59	298.10±6.12	307.57±7.23	303.10±2.60	297.47±2.95
D3	3:1	309.53±12.11	303.17±1.79	303.17±2.24	307.07±3.01	296.63±14.20	315.27±4.35	303.63±5.80	301.67±4.00
D4	5:0.5	299.63±2.68	305.27±5.35	301.90±4.69	307.83±7.86	301.80±4.63	306.97±9.92	306.80±2.52	296.60±10.82
D5	4:0.5	298.30±9.54	312.23±8.79	300.20±5.38	302.07±7.07	300.87±6.07	303.33±9.08	302.00±4.92	304.40±5.03
D6	3:0.5	305.50±3.51	306.30±4.75	303.97±5.26	303.90±6.16	298.87±2.10	311.37±1.57	305.77±5.16	296.60±13.67
D7	5:0	311.00±10.65	303.30±3.74	299.90±5.94	296.87±3.19	301.10±3.52	309.67±10.74	305.10±11.21	298.57±3.95
D8	4:0	316.77±12.86	307.23±2.73	312.70±11.25	311.80±15.12	294.20±11.09	307.63±3.01	306.57±9.48	299.87±3.26
D9	3:0	317.40±7.64	303.90±6.44	305.13±1.36	299.57±2.67	300.17±2.87	310.03±5.66	307.20±10.67	303.70±4.62

Table 23. Osmolarity of D1-D9 before and after storage at 4 ºC (mean ± S.D., n=3)

No	Ratio	Osmolarity (mOsmol)							
		Initial	After 4 day	After 7 day	After 10 day	After 14 day	After 21 day	After 1 month	After 2 month
D1	5:1	306.37±3.46	305.60±6.58	297.80±0.61	298.47±7.06	299.13±6.17	292.90±7.99	301.27±3.53	300.03±5.02
D2	4:1	311.13±4.30	306.07±3.78	289.33±2.10	301.37±4.76	303.07±3.50	300.17±5.06	305.00±3.40	298.27±3.70
D3	3:1	309.53±12.11	304.03±5.64	296.40±2.25	301.17±4.67	302.10±3.55	300.03±6.27	305.47±2.10	302.00±9.24
D4	5:0.5	299.63±2.68	306.23±8.38	294.80±2.56	300.40±8.23	302.73±4.68	297.90±11.29	299.07±3.16	303.27±10.05
D5	4:0.5	298.30±9.54	307.47±8.87	300.93±9.03	297.83±5.51	305.07±6.69	297.60±5.72	299.83±6.16	300.13±2.99
D6	3:0.5	305.50±3.51	305.03±12.19	298.40±2.89	301.03±3.13	304.77±12.20	302.40±6.58	303.80±9.92	297.70±7.57
D7	5:0	311.00±10.65	307.57±7.09	299.97±5.88	301.67±10.93	307.97±8.98	298.33±3.55	300.23±2.28	304.87±14.20
D8	4:0	316.77±12.86	311.30±6.32	303.93±10.56	301.97±7.81	306.77±8.06	297.40±3.15	304.37±5.26	307.17±7.48
D9	3:0	317.40±7.64	308.07±2.15	299.57±5.22	298.30±7.14	302.03±8.04	297.50±7.89	305.13±4.46	309.03±7.80

Table 24. Mean particle size of D1-D9 before and after storage at room temperature (mean ± S.D., n=3)

No	Ratio	Mean particle size (nm)							
		Initial	After 4 day	After 7 day	After 10 day	After 14 day	After 21 day	After 1 month	After 2 month
D1	5:1	17.20±0.30	18.20±1.13	20.93±4.26	19.57±2.50	19.83±2.21	20.07±3.05	20.00±2.23	18.93±1.96
D2	4:1	17.53±0.06	18.93±1.07	20.27±3.12	19.20±2.17	20.00±2.23	20.80±2.96	20.10±2.70	19.50±2.25
D3	3:1	17.37±0.25	18.53±1.02	19.83±2.08	18.90±1.91	21.73±5.00	20.60±2.88	20.40±2.17	19.40±2.00
D4	5:0.5	17.33±0.29	18.70±0.92	19.57±1.80	19.37±2.56	19.83±2.55	20.13±2.70	20.37±2.32	19.50±2.40
D5	4:0.5	17.53±0.38	18.83±0.68	19.47±1.97	18.97±2.29	19.80±2.48	20.37±1.71	20.53±1.86	19.63±2.31
D6	3:0.5	17.30±0.10	18.07±0.90	19.73±2.32	19.30±2.49	19.97±2.53	21.00±2.78	20.63±1.86	19.07±1.31
D7	5:0	17.27±0.31	19.03±1.08	20.27±2.71	18.20±3.30	20.10±3.64	20.80±3.01	21.17±2.66	19.47±2.47
D8	4:0	17.73±0.15	18.47±0.95	19.97±2.10	18.80±2.19	20.07±3.90	20.73±2.75	21.03±1.17	19.27±0.81
D9	3:0	17.50±0.26	18.13±1.18	19.43±2.15	18.77±1.92	20.33±3.34	20.80±2.72	20.80±2.29	18.53±1.40

Table 25. Mean particle size of D1-D9 before and after storage at 4 ºC (mean ± S.D., n=3)

No	Ratio	Mean particle size (nm)							
		Initial	After 4 day	After 7 day	After 10 day	After 14 day	After 21 day	After 1 month	After 2 month
D1	5:1	17.20±0.30	18.77±1.27	19.50±1.78	19.83±2.72	19.70±2.31	19.30±1.01	21.33±2.72	18.47±1.82
D2	4:1	17.53±0.06	17.90±0.46	19.67±1.01	19.90±2.36	20.83±3.12	19.50±0.75	21.03±2.54	18.33±2.50
D3	3:1	17.37±0.25	17.97±0.57	20.23±0.99	19.70±1.85	21.20±3.34	19.63±1.06	20.83±2.49	18.97±2.44
D4	5:0.5	17.33±0.29	19.07±0.84	19.70±0.95	19.53±1.81	21.90±3.86	18.80±0.72	20.73±2.49	18.63±3.00
D5	4:0.5	17.53±0.38	18.70±0.35	19.60±0.17	19.47±2.21	21.27±2.27	19.73±1.05	20.87±2.29	18.80±2.46
D6	3:0.5	17.30±0.10	18.37±0.21	18.50±0.85	19.40±2.29	21.37±1.92	19.37±1.11	20.80±2.72	18.03±3.31
D7	5:0	17.27±0.31	18.70±0.72	18.23±0.64	19.33±2.25	21.00±2.26	19.20±1.05	21.50±2.00	18.07±3.06
D8	4:0	17.73±0.15	18.47±0.60	18.90±0.78	19.00±1.80	21.87±1.90	19.33±0.71	21.13±3.15	18.50±1.93
D9	3:0	17.50±0.26	18.20±0.82	18.50±0.61	18.17±1.70	21.73±1.16	19.53±1.00	20.40±3.29	19.27±0.74

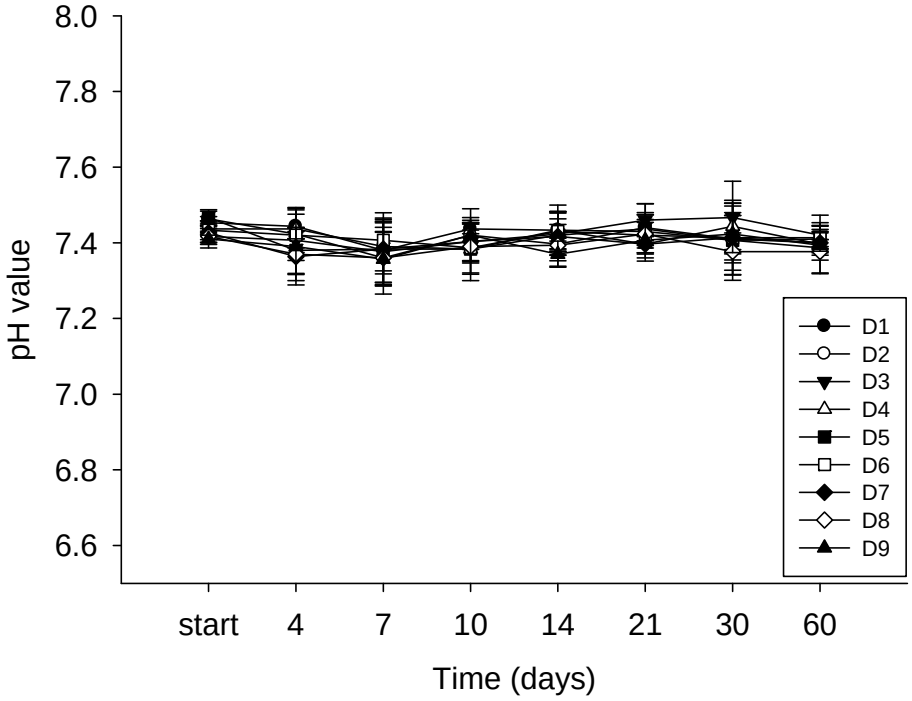

Fig 11. pH profile of D1-D9 stored at room temperature

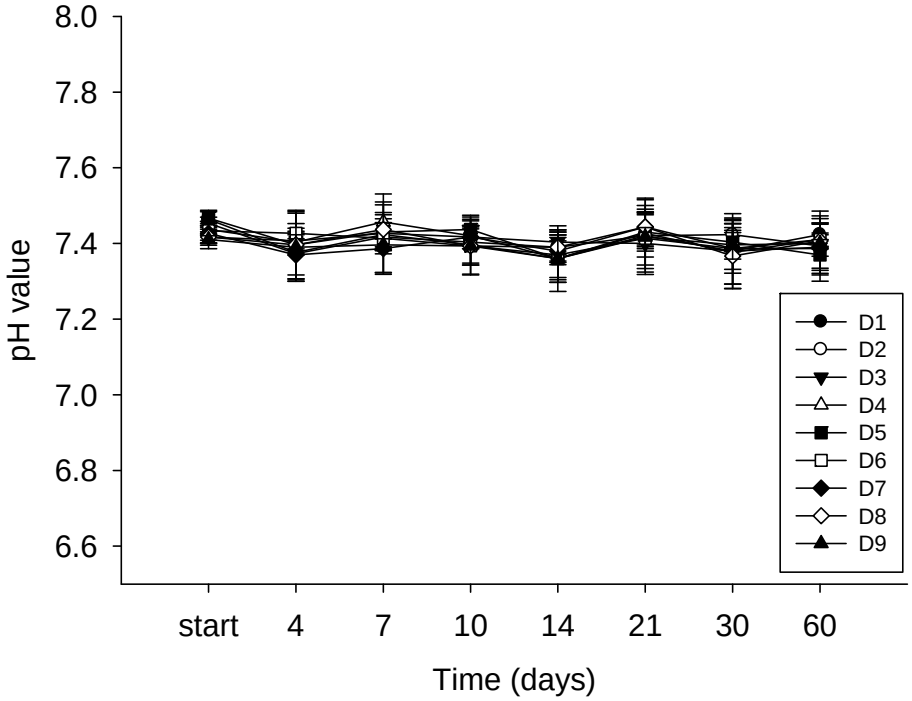

Fig 12. pH profile of D1-D9 stored at 4ºC

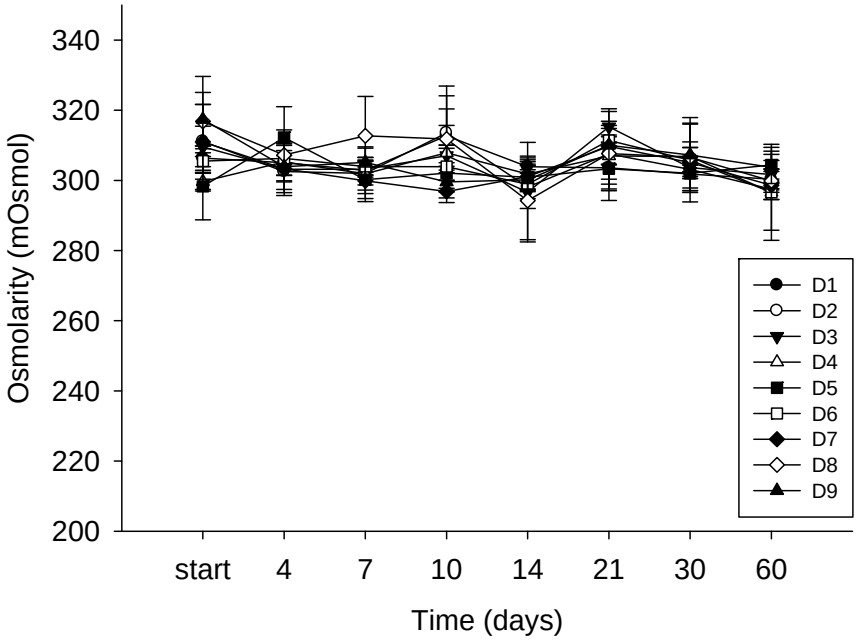

Fig 13. Osmolarity profile of D1-D9 stored at room temperature

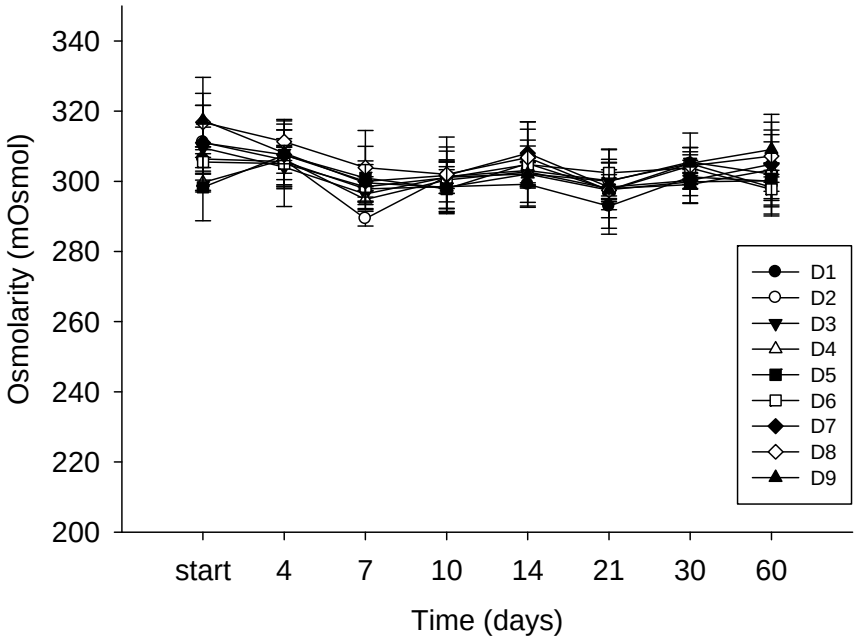

Fig 14. Osmolarity profile of D1-D9 stored at 4ºC

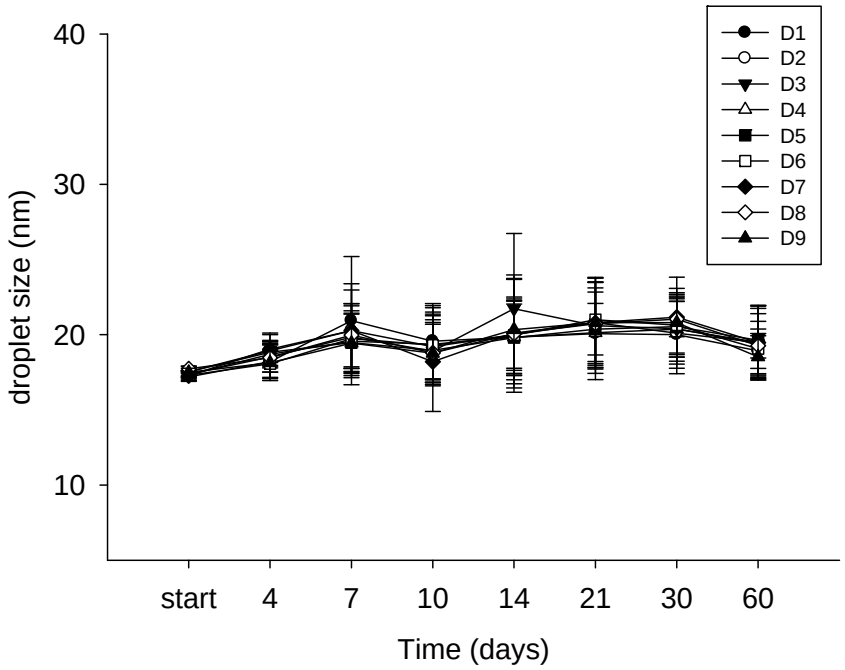

Fig 15. Droplet size profile of D1-D9 stored at room temperature

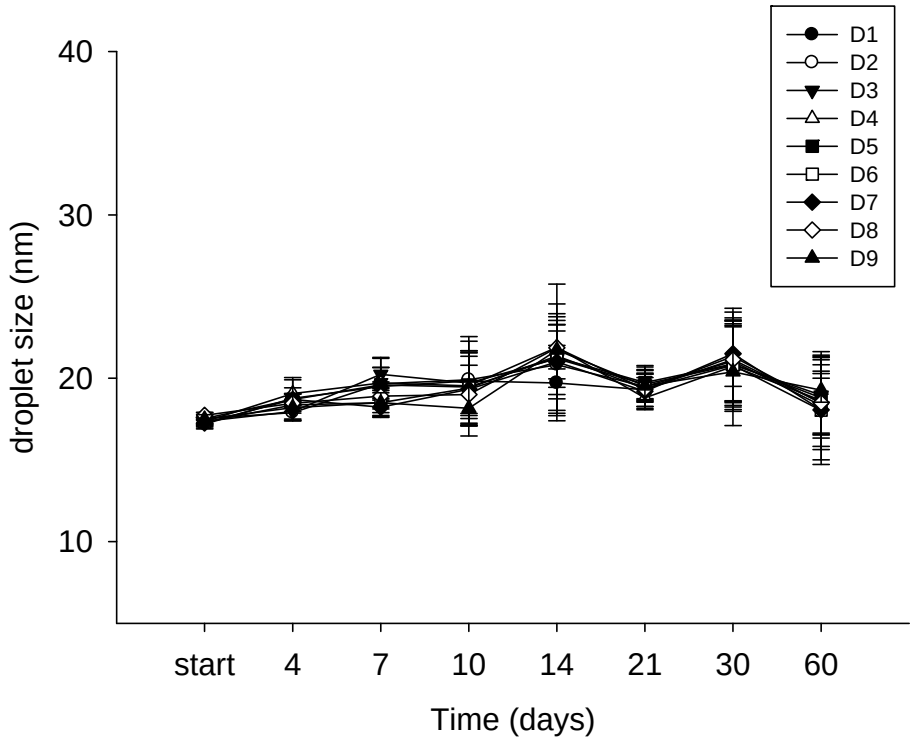

Fig 16. Droplet size profile of D1-D9 stored at 4ºC

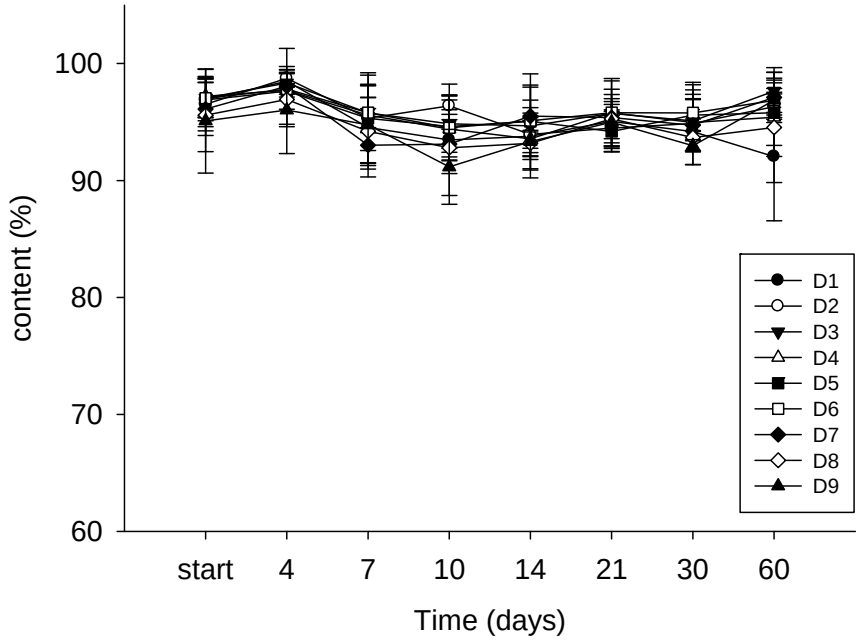

Fig 17. Content profile of D1-D9 stored at room temperature

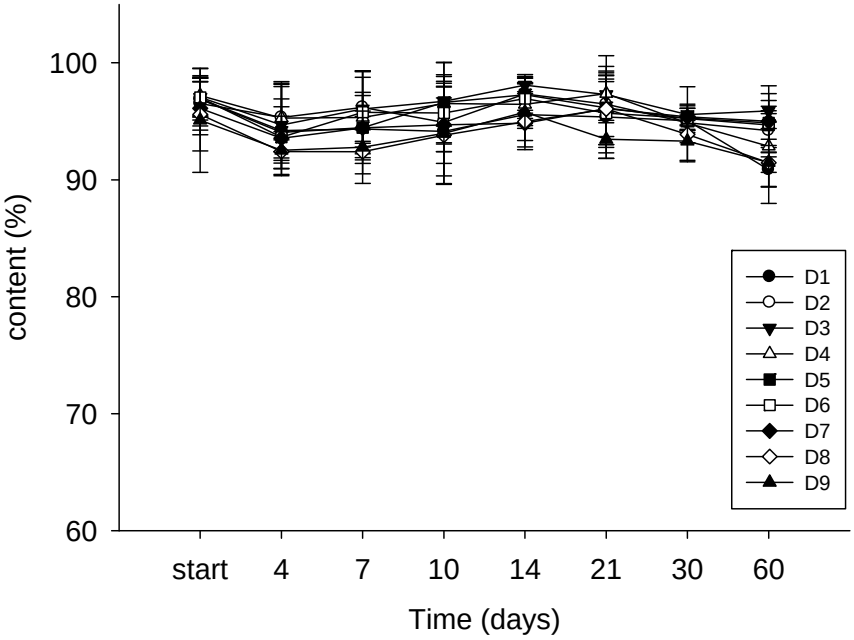

Fig 18. Content profile of D1-D9 stored at 4ºC

3.5. Schirmer tear test

In this study, we investigated the induced dry eye and acceleration of lacrimation by CsA eye drops.

The Schirmer tear test scores (reported as millimeters of wet strip after 2 min of insertion) obtained treatment with AS are presented in **table 26.** and **Figure 19.**. 1% AS solution administered rabbit one eye for causing dry eye . And the other eye was administered normal saline as a control body. A decreased tear production was observed in treated with AS eye during the experiment. On the first day, after administration of the normal saline or AS solution, the gap between the control and AS treated eye was insignificant, control was 10.08 ± 3.40 and treated eye was 9.94 ± 3.19. As time passes, the difference of two body was distinct and grew. The last test point, the gap between two body was 3.45 ± 1.59. This tear difference value was about twenty fold of first test point score gap value, 0.14 ± 1.59.

The Schirmer tear test scores relevant to the treatment with the formulation under test are illustrated in **Table 27.** and **Figure 20.**. Group 2 and group 3 were to make sure whether is treatment of dry eye. Both eyes of all groups were administered 1% AS solution for causing dry eye. Then one eye treated drug formulation and another treated normal saline. Drug formulation was D1; Cremophor EL 5% (group 2) and commercial CsA emulsion (group 3). On the first day, after administration, the gap between the control with AS and treated eye were similar in two groups, group 2 (0.71 ± 3.17) and group 3 (0.69 ± 2.17). But after that, the tear difference value between group 2 and group 3 was distinct. Especially, at the last test point, the tear difference value of group 2 was 7.28 ± 4.33 and that of

group 3 was 2.09 ± 1.24. This means tear difference value of group 2 was about 3 and 1/2 fold of that of group 3.

In sum, drug formulation of this study was effect compare with the commercial CsA emulsion.

Table 26. The Schirmer tear test score with induced dry eye

Group 1	day 1	day 5
Control (n=8)	10.08 ± 3.40	12.18 ± 3.85
Treated eye-AS (n=8)	9.94 ± 3.19	8.73 ± 2.69
Tear difference value	0.14 ± 1.59	3.45 ± 1.59

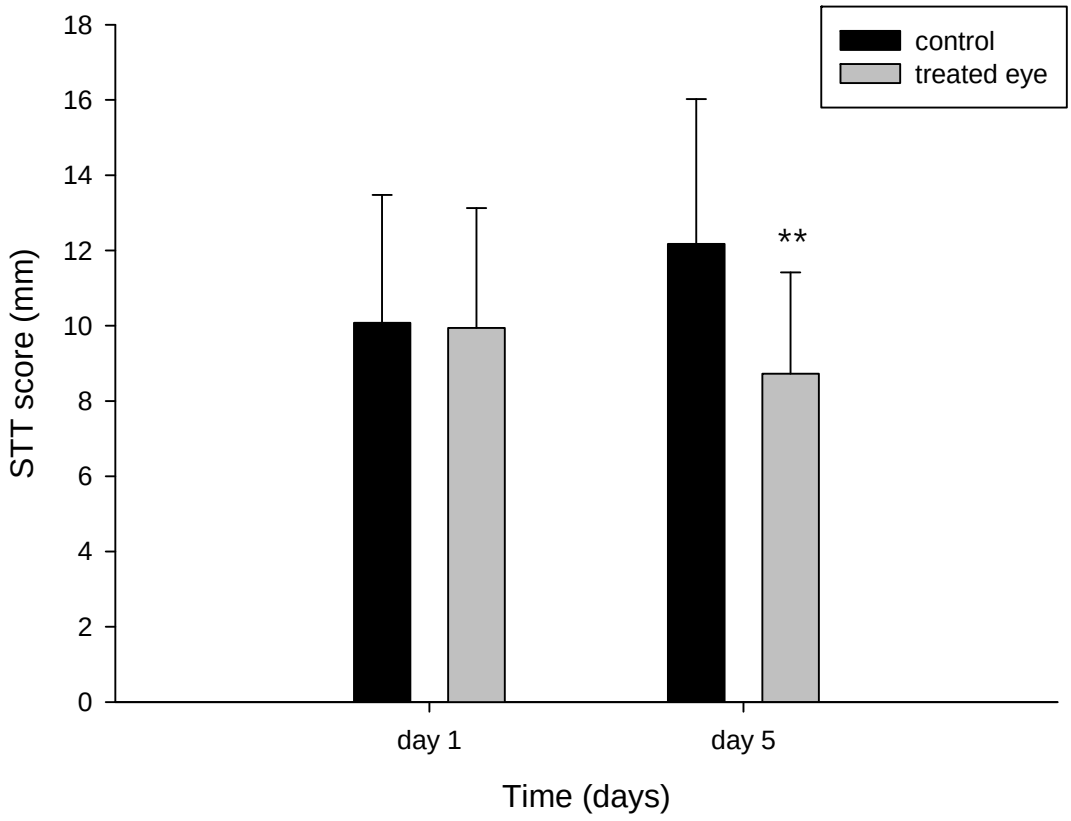

Fig 19. The Schirmer tear test score with induced dry eye
Statistically differences were found in the study group at day 5 when compared with the day 1. Data show mean ± SD (error bars). $^{**}P < 0.005$.

Table 27. The Schirmer tear test score with cure

Group 2	day 1	day 3	day 5
Control with AS (n=8)	12.54 ± 2.82	9.06 ± 2.21	7.38 ± 1.94
Treated eye-D1 (n=8)	13.25 ± 3.53	12.28 ± 2.26	14.65 ± 3.85
Tear difference value	0.71 ± 3.17	3.21 ± 1.99	7.28 ± 4.33
Group 3	day 1	day 3	day 5
Control with AS (n=8)	9.44 ± 2.76	7.59 ± 2.80	7.81 ± 2.37
Commercial CsA (n=8)	10.13 ± 2.26	9.44 ± 3.74	9.90 ± 3.21
Tear difference value	0.69 ± 2.17	1.85 ± 1.89	2.09 ± 1.24

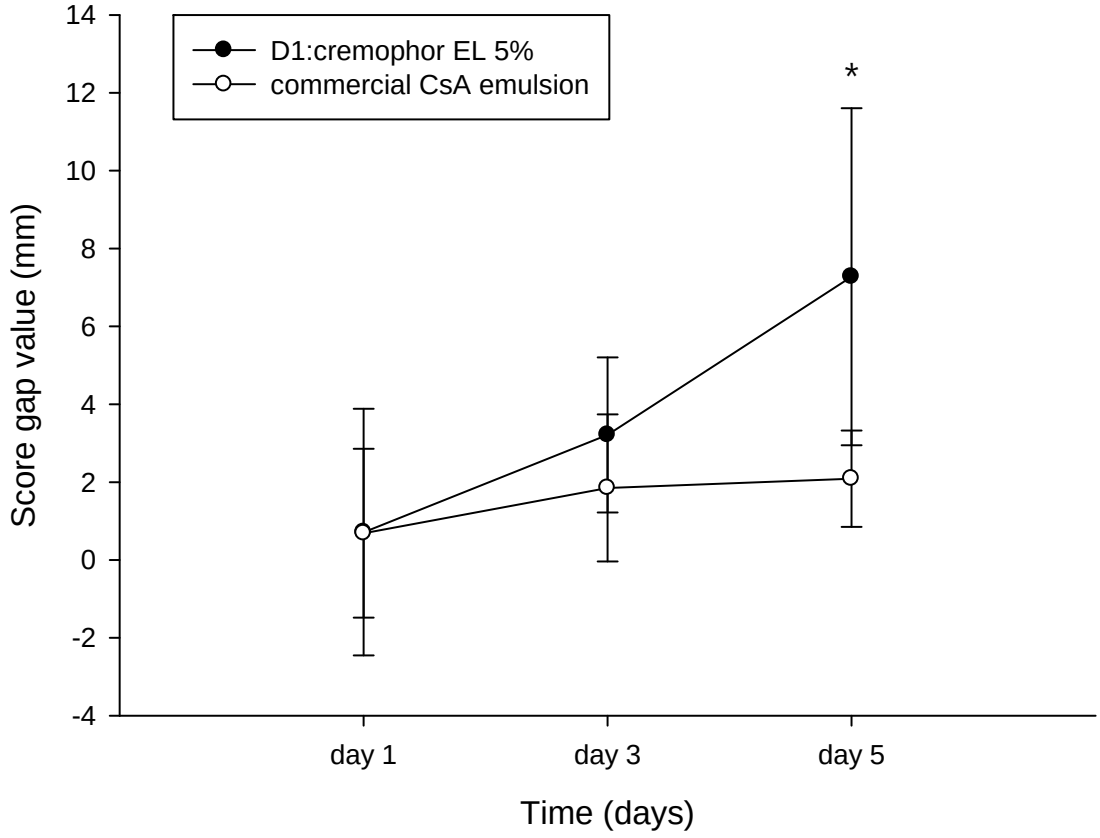

Fig 20. The Schirmer tear test score with cure
Statistically differences were found in the study group at day 5 when compared with the day 1 and day 3. Data show mean ± SD (error bars). $^{*}P < 0.05$

3.6. Goblet cell density and conjunctival epithelial morphology

Topically applied cyclosporine A was rapidly absorbed into the ocular tissues known to be affected in dry eye disease (the conjunctiva, cornea, and lachrymal gland) at concentrations sufficiently high to suppress inflammatory processes (Acheampong et al. 1999, 91-103). Especially, goblet cells, located in the surface of the conjunctiva, secreted mucin. Once mucin is secreted by goblet cells, it has the capability to hydrate and gel, thus keeping the conjunctiva moist. This film produces a protective covering over the ocular surface shielding it from a variety of pathogens, chemicals, and environmental toxins.

Exposure of normal mice to a low-humidity environment in a controlled environment chamber can lead to significant alterations in tear secretion, goblet cell density, and acquisition of dry eye related ocular surface signs (Barabino et al. 2005, 2766-71). CsA emulsion, but not artificial tears, increases goblet cell density in the bulbar conjunctiva in patients with dry eye (Pflugfelder et al. 2008, 64-9).

To evaluate the effects of sequential treatment with atropine sulfate or CsA micelle solutions on conjunctival goblet cell density in rabbit, samples of conjunctiva sac were stained with H& E solution.

The number of goblet cells in the conjunctiva in the AS exposed rabbits (41.67 ± 7.94) was significantly lower than in control rabbits (83.33 ± 16.50) at goup 1. The number of goblet cells in the conjunctiva with D1 or commercial CsA were significantly higher than the number of goblet cells in the conjunjuctiva with control with AS.

As shown in **Figure 23.**, goblet cells are stained *purple* and are located in the superficial epithelium. Conjunctiva of rabbits after 5 days of treatment with AS were (B), (C) and (E). These epithelium was thinned with loss of goblet cells. However, conjunctiva of rabbits after 5 days of treatment with normal saline (A) were composed of sufficient layers of epithelial cells and contains numerous goblet cells. And conjunctiva of rabbits after 5 days of treatment with D1 (D) and with commercial CsA emulsions (F) were recovered epithelium and relatively increased the number of goblet cells.

Table 28. Average conjunctival goblet cell density

Group 1	Goblet cell density
Control	83.33 ± 16.50
Treated eye-AS	41.67 ± 7.94
Group 2	Goblet cell density
Control with AS	45.83 ± 7.03
Treated eye-D1	94.83 ± 8.38
Group 3	Goblet cell density
Control with AS	37.17 ± 10.94
Commercial CsA	65.17 ± 11.51

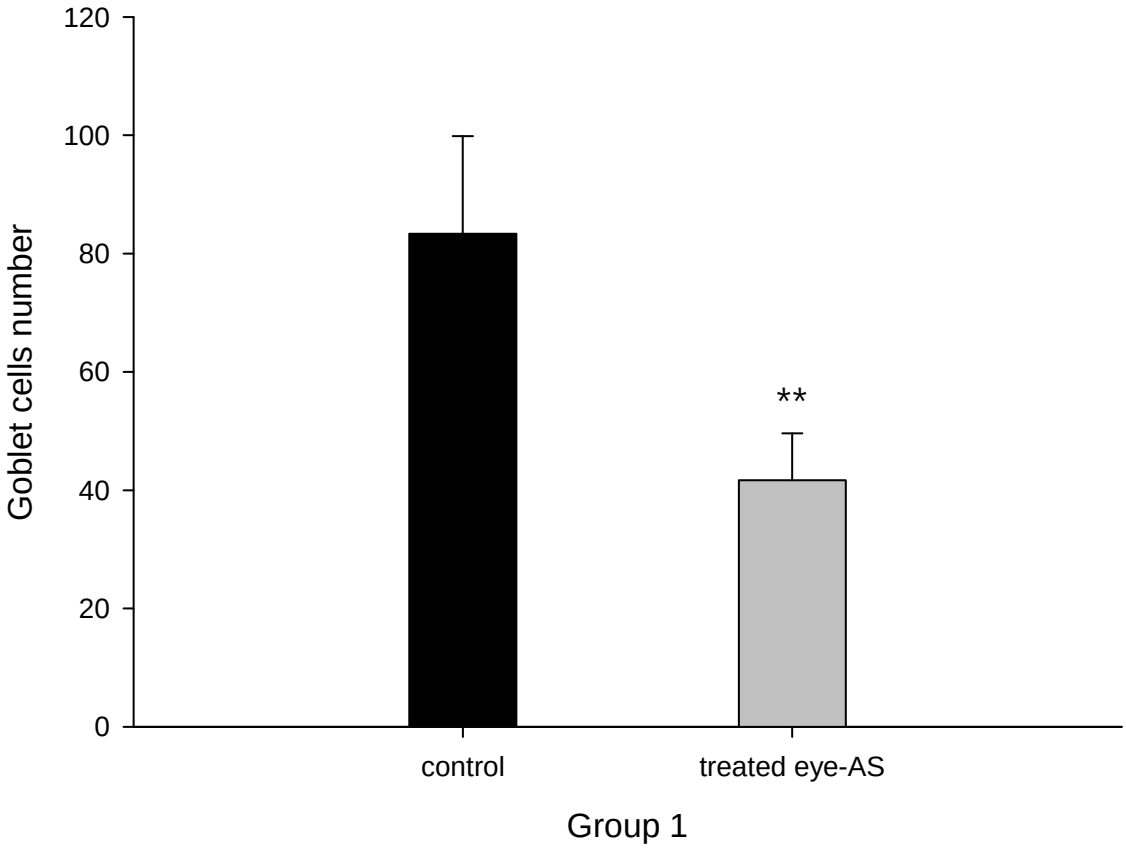

Fig 21. Average conjunctival goblet cell density in group 1
The mean ± SD number of goblet cells in three separate x40 microscopic fields in the conjunctiva. The *t*-test was used for statistical comparisons. $^{**}P < 0.005$

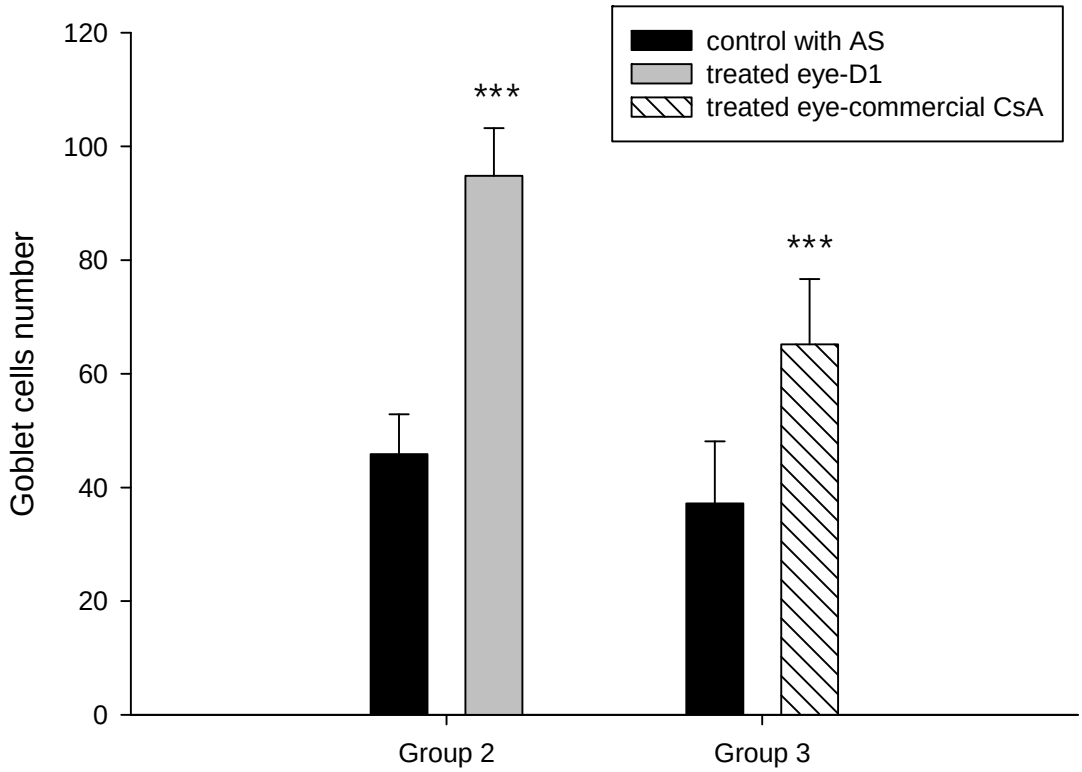

Fig 22. Average conjunctival goblet cell density in group 2 and group 3
The mean ± SD number of goblet cells in three separate x40 microscopic fields in the conjunctiva. The *t*-test was used for statistical comparisons. $^{***}P < 0.0005$

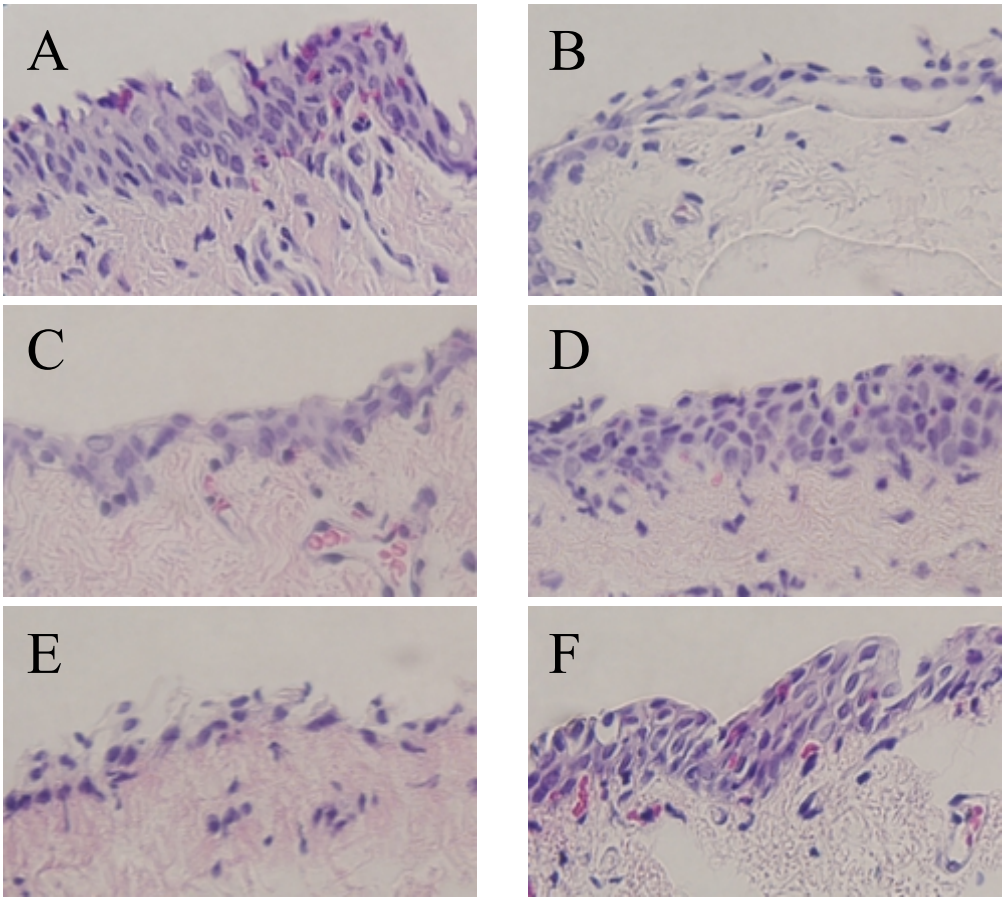

Fig 23. Histologic section of paraffin embedded rabbit conjunctiva stained with H&E solution
(A) Conjunctiva of administration normal saline. (group 1)
(B) Conjunctiva of administration atropine sulfate. (group 1)
(C) Conjunctiva of administration NS after administration AS. (group 2)
(D) Conjunctiva of administration D1 after administration AS. (group 2)
(E) Conjunctiva of administration NS after administration AS. (group 3)
(F) Conjunctiva of administration commercial CsA emulsions after administration AS. (group 3)
Magnification, x 40

4. Conclusion

In this study we investigated the potential of a micelle solution containing nonionic surfactant Cremophor EL for ocular delivery of CsA to ocular tissue. To selected formulation of micelle solution, compositions of drug were measured (pH, osmolarity, size). Selected micelle formulations were stable for a long period of time. Compared with commercial CsA emulsion, the CsA micelle formulations were good in dry eyes. In conclusion, CsA micellar delivery system for dry eyes can offer the great stability and therapeutic efficacy. Furthermore, CsA micellar delivery system is an advantageous technique for shorther processing time and scale-up.

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ABSTRACT

Cyclosporine A micellar delivery system for dry eyes

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The aim of this thesis was to develop ophthalmic cyclosporine A micellar delivery system. Cyclosporine A (CsA) is not only an immunosuppressant but also a drug for improving tear production in patients with keratoconjunctivitis sicca, Sjörgren syndrome and dry eye syndrome. But it is difficult to make the stable CsA ophthalmic drug due to the relatively high molecular weight (1202.61) and poor solubility.

The CsA micellar delivery systems were prepared only by mixing and homogenizing. Cremophor EL as surfactant and ethanol as cosolvent were chosen by solubility test. pH, osmolarity, particle size and therapeutic efficacy were evaluated. Droplet size was assessed to check critical micelle concentration (CMC) in various micelle formulations. If CsA were sufficiently wetted, micelle

formulations were observed about 14nm on size when concentration of Cremophor EL was more than 1%.

The stability of CsA micelle solution was assessed as a function of pH, and concentration of Cremophor EL and ethanol by pH meter, osmometer, electrophoretic light scattering spectrophotometer (ELS-Z) and high-performance liquid chromatography (HPLC). These micelle solutions had osmolarity of about 280mOsmol to 320mOsmol and mean size of about 15nm to 25nm. The significant change of pH, osmolarity, droplet size and content measurement are not shown. Also, other visible changes like the aggregation or the phase separation were not shown.

Schirmer tear test with New Zealand albino rabbits showed that micelle formulation of this study was more effective comparing with the commercial CsA emulsion on the increasing tear production. Similar with previous commercial formulation, recovery of goblet cell density and epithelium was observed in H&E staining.

In conclusion, CsA micellar delivery system for dry eyes can offer a great stability and therapeutic efficacy. Furthermore, CsA micellar delivery system is an advantageous technique for shorter processing time and scale-up.

Key words : cyclosporine A, surfactant, droplet size, micellar delivery system