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Effects of chondrocyte-derived extracellular matrix in a dry eye mouse model

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Purpose: The occurrence of repetitive dry eye is accompanied by inflammation. This study investigated the anti-inflammatory effects of chondrocyte-derived extracellular matrix (CDECM) on the cornea and conjunctiva in a dry eye mouse model.

Methods: Dry eyes were experimentally induced in 12- to 16-week-old NOD.B10.*H2*^{b} mice (Control) via subcutaneous injections of scopolamine (muscarinic receptor blocker) and exposure to an air draft for 10 days (desiccation stress [DS] 10D group). Tear volume and corneal smoothness were measured at 3, 5, 7, and 10 days after the instillation of PBS (PBS group) or CDECM (CDECM group). The corneas and conjunctivas were sectioned and stained with hematoxylin and eosin (H&E) and periodic acid Schiff (PAS). The expression of inflammatory markers (i.e., tumor necrosis factor-α [TNF-α], matrix metalloproteinase-2 [MMP-2], MMP-9, intercellular adhesion molecule-1 [ICAM-1], and vascular cell adhesion molecule-1 [VCAM-1]) was detected by quantitative real-time (qRT)-PCR and western blotting. All data were statistically processed using SPSS version 18.0.

Results: The instillation of CDECM after the removal of the DS increased tear production by up to 3.0-fold, and corneal smoothness improved to 80% compared to the PBS group ($p<0.05$). In the CDECM group, the detachment of the corneal epithelial cells was reduced by 73.3% compared to the PBS group, and the conjunctival goblet cell density was significantly recovered to the control levels ($p<0.05$). The expression of inflammatory factors was decreased in the cornea and conjunctiva of the CDECM group compared to the PBS group.

Conclusions: These observations suggest that CDECM induced effective anti-inflammatory improvements in the cornea and conjunctiva in this experimental model of dry eye.

The prevalence of dry eye increases with age; it has been reported to be 6% among 40 year olds, increasing to 25% among those over 65 years of age [1]. Dry eye is caused by an imbalance between tear-related components due to factors such as reduced tear production and the excessive evaporation of tears from the ocular surface. The symptoms of dry eye include blurred vision, photophobia (light sensitivity), and eye irritation, including feelings of irritation and dryness. Thus, dry eye contributes to increased discomfort or difficulty in performing normal tasks such as driving, reading, and watching television [2,3].

The tear film is composed of a lipid layer, an aqueous layer, and a mucous layer and covers the ocular surface. Instability of the tear film layers can be caused by dry eye disease in the ocular surface, leading to serious disabilities of the cornea and conjunctiva. The pathologies of the ocular surface that can be related to dry eye include increased permeability of the corneal epithelium, loss of conjunctival goblet cells, and deformation of the conjunctiva. Furthermore, dry eye can lead to disorders of the corneal epithelium, corneal ulcers, and erosion of the corneal epithelium that can be severe enough to require corneal transplants in some cases [4,5].

Prolonged dry eye has been reported to induce inflammation of the ocular surface, increase the levels of inflammatory cytokines in the conjunctival epithelium and tear fluid, increase the expression of adhesion molecules in the conjunctival epithelium, and increase the numbers of T lymphocytes in the conjunctiva [6-9]. Moreover, patients with keratoconjunctivitis sicca (KCS) have been reported to exhibit increased matrix metalloproteinase (MMP) and inflammatory cytokine activities in the tear fluid and conjunctival epithelia [6,7,10,11].

Currently, the most popular treatment for dry eye is the prescription of topical artificial tear solutions that contain a viscoelastic compound such as methylcellulose, chondroitin sulfate, or hyaluronic acid. However, the efficacies of these compounds are limited because they are physically and physiologically different from mucins [12-14]. Various other studies have reported the treatment of dry eye and associated inflammation with cyclosporine A (the only Food and Drug Administration [FDA]-approved therapeutic agent), topical

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corticosteroids, tetracyclines, macrolides, and omega-3 and omega-6 fatty acids [15-21]. However, cyclosporine A appears to be an insufficient treatment due to adverse reactions such as stinging and hypersensitivity, as well as drug resistance problems that occur in some patients with dry eye. Moreover, long-term treatment with corticosteroids can cause serious side effects such as hypertension, glaucoma, cataracts, and infections [22].

Extracellular matrix (ECM) scaffolds are known to be repositories of cytokines and growth factor receptors and provide environments that are similar to those of the natural tissue by recreating the interactions with cell surface receptors [23,24]. In addition, ECM scaffolds have been used to regenerate blood vessels, bladders, tendons, cartilage, and corneal tissue, and the composition and structures of the ECM scaffolds influence the phenotype, attachments, migration, and proliferation of cells [25-28]. Chondrocyte-derived ECM (CDECM) has been used to decrease adhesions, primarily in orthopedic surgery [29,30]. CDECM scaffolds not only provide good environments for cartilage tissue formation but also suppress vessel invasion and angiogenesis in vivo [31-33]. Yang et al. reported that the use of an ECM membrane reduced postoperative adhesion around the surgical areas following strabismus surgeries in rabbits [34]. Moreover, CDECM has been shown to suppress lesion growth, corneal neovascularization, and pathogenesis in the pterygium [35]. Lee et al. observed that CDECM significantly suppressed neovascularization from the cornea in a rabbit model of corneal alkaline burns [36]. Another study demonstrated that CDECM inhibited the angiogenesis, adhesion, and proliferation of endothelial cells and prevented neovascularization in a rabbit model of suture-induced corneal neovascularization [37]. However, the effects of CDECM in the cornea and conjunctiva on the inflammation associated with dry eye have not been investigated.

In the present study, we investigated the effects of CDECM on tear production and the ocular surface changes after desiccation stress (DS) in an experimental mouse model of dry eye. Experimental dry eye was induced in the NOD.B10.*H2*^{b} strain through the pharmacological inhibition of aqueous tear production and exposure to desiccating environmental stress. After the removal of the desiccating environmental and pharmacological stresses, we analyzed the changes in tear production, ocular surface irregularities, corneal epithelial permeability, conjunctival goblet cells, and the expression of inflammatory factors in the corneal and conjunctival tissue.

METHODS

The mouse model of dry eye and experimental procedures: The NOD.B10.*H2*^{b} mice were purchased from Jackson Laboratory (Bar Harbor, ME). This study was performed according to the guidelines for animal experiments of the Inje University College of Medicine (No. 2012–053) and the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. One hundred and five 12- to 16-week-old male NOD.B10.*H2*^{b} mice underwent DS via exposure to an air draft from a fan at an ambient humidity of 30–40% for 18 h per day for 10 days, as well as a subcutaneous 0.2 ml injection of 0.5 mg/0.2 ml scopolamine hydrobromide (Sigma-Aldrich, St. Louis, MO) [38,39] into alternating hindquarters four times per day (9 AM, 12 PM, 3 PM, and 6 PM), as previously reported. As a parasympathetic nerve inhibitor, scopolamine suppresses the central nervous system and has pharmacological effects similar to atropine, such as mydriasis, nerve paralysis, and inhibited secretions. During these experiments, the animals' behavior and food and water intake were not restricted. The success rate of this experimental dry eye model was 60% (63 of the 105 total animals) in the normal NOD.B10.*H2*^{b} mice (Table 1). The PBS or CDECM eye drops in the experimental dry eye mice were only used to satisfy two conditions, as follows: there was less than 0.029 µl of tear production and an irregularities score of more than two points (Table 2) [40]. The 10 mg/ml concentration of the CDECM eye drop was prepared by dissolving the CDECM film in PBS. First, the CDECM film was manufactured in the following manner: Primary chondrocytes from porcine knee joints were expanded in monolayer culture and then cultured in a monolayer for 3 weeks [33]. The resulting monolayer was treated with 200 U/ml DNase I and washed thoroughly three times with Dulbecco's PBS 1X (8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, 0.24 g of KH_2PO_4, pH 7.4; Gibco, Carlsbad, CA). The CDECM film was then cut with a biopsy punch into circles of 8 mm in diameter, and the pieces of CDECM film were dissolved in PBS. The eye drops were instilled at 10 mg/ml five times per day (9 AM, 11 AM, 1 PM, 3 PM, and 5 PM) for 10 days.

The following six experimental groups were evaluated: control (normal, n=20); DS 10D (untreated, DS for 10 days, n=19); PBS 5D (n=3, 5 µl/eye instilled bilaterally five times per day for 5 days after the removal of the DS); PBS 10D (n=19, 5 µl/eye instilled bilaterally five times per day for 10 days after the removal of the DS); CDECM 5D (n=3, 5 µl/eye instilled bilaterally at 10 mg/ml five times per day for 5 days after the removal of the DS); and CDECM 10D (n=19, 5 µl/eye instilled bilaterally at 10 mg/ml five times per day

Table 1. The success rate of the experimental dry eye model.

DS 10D (n = 105)											
No.	TV (μl) R/L	CIS R/L	Dry eye	No.	TV (μl) R/L	CIS R/L	Dry eye	No.	TV (μl) R/L	CIS R/L	Dry eye
1*	**0.016/0.022**	**4/2**	**Yes**	**36†**	**0.019/0.022**	**3/3**	**Yes**	**71§**	**0.029/0.022**	**3/3**	**Yes**
2	0.091/0.122	0/1	No	**37†**	**0.022/0.029**	**4/3**	**Yes**	**72§**	**0.025/0.019**	**2/4**	**Yes**
3*	**0.029/0.025**	**3/3**	**Yes**	**38†**	**0.029/0.025**	**3/3**	**Yes**	**73§**	**0.019/0.022**	**2/4**	**Yes**
4*	**0.029/0.029**	**4/3**	**Yes**	**39†**	**0.029/0.029**	**3/3**	**Yes**	**74§**	**0.025/0.022**	**3/3**	**Yes**
5	0.091/0.060	1/0	No	40	0.122/0.091	0/0	No	75	0.091/0.091	0/0	No
6*	**0.022/0.029**	**3/4**	**Yes**	41	0.122/0.060	0/1	No	**76§**	**0.022/0.029**	**3/3**	**Yes**
7*	**0.025/0.029**	**3/3**	**Yes**	**42†**	**0.022/0.029**	**3/3**	**Yes**	**77§**	**0.029/0.022**	**4/2**	**Yes**
8*	**0.022/0.025**	**3/3**	**Yes**	**43†**	**0.029/0.025**	**4/3**	**Yes**	**78§**	**0.022/0.019**	**3/3**	**Yes**
9*	**0.029/0.025**	**3/3**	**Yes**	44	0.091/0.060	0/0	No	79	0.122/0.091	0/0	No
10	0.122/0.091	0/0	No	**45†**	**0.019/0.022**	**2/4**	**Yes**	**80§**	**0.029/0.013**	**3/5**	**Yes**
11*	**0.019/0.022**	**3/3**	**Yes**	46	0.091/0.091	0/0	No	**81§**	**0.013/0.029**	**3/3**	**Yes**
12*	**0.025/0.029**	**3/3**	**Yes**	47	0.122/0.060	0/1	No	82	0.060/0.075	1/1	No
13	0.060/0.122	0/0	No	48	0.091/0.060	0/0	No	**83§**	**0.029/0.029**	**3/3**	**Yes**
14	0.091/0.091	0/0	No	**49†**	**0.019/0.025**	**2/3**	**Yes**	84	0.091/0.060	0/1	No
15	0.122/0.060	0/1	No	**50†**	**0.029/0.029**	**3/3**	**Yes**	85	0.122/0.060	0/0	No
16*	**0.029/0.029**	**3/4**	**Yes**	51	0.060/0.075	0/0	No	**86§**	**0.029/0.013**	**4/3**	**Yes**
17*	**0.029/0.013**	**4/3**	**Yes**	52	0.075/0.060	1/1	No	87	0.060/0.091	0/1	No
18*	**0.029/0.013**	**3/5**	**Yes**	**53†**	**0.029/0.029**	**4/3**	**Yes**	**88§**	**0.029/0.029**	**3/3**	**Yes**
19	0.075/0.060	0/0	No	**54†**	**0.029/0.029**	**3/4**	**Yes**	89	0.060/0.091	1/1	No
20*	**0.013/0.013**	**3/3**	**Yes**	55	0.060/0.060	1/0	No	**90§**	**0.013/0.029**	**3/3**	**Yes**
21	0.060/0.075	1/0	No	**56†**	**0.013/0.029**	**5/4**	**Yes**	**91§**	**0.029/0.013**	**4/5**	**Yes**
22*	**0.029/0.013**	**4/3**	**Yes**	57	0.075/0.060	0/0	No	92	0.075/0.060	1/0	No
23*	**0.029/0.029**	**4/5**	**Yes**	**58†**	**0.013/0.029**	**3/4**	**Yes**	**93§**	**0.029/0.013**	**3/4**	**Yes**
24	0.075/0.060	1/1	No	**59†**	**0.029/0.013**	**5/3**	**Yes**	**94§**	**0.013/0.029**	**3/5**	**Yes**
25	0.060/0.075	1/1	No	60	0.106/0.060	0/1	No	95	0.091/0.060	1/1	No
26*	**0.029/0.029**	**3/5**	**Yes**	**61†**	**0.029/0.013**	**4/3**	**Yes**	**96§**	**0.013/0.013**	**4/5**	**Yes**
27	0.075/0.060	0/0	No	**62†**	**0.013/0.013**	**5/4**	**Yes**	**97#**	**0.022/0.019**	**3/3**	**Yes**
28*	**0.029/0.029**	**3/3**	**Yes**	**63†**	**0.013/0.029**	**3/5**	**Yes**	**98#**	**0.029/0.029**	**4/3**	**Yes**
29*	**0.013/0.013**	**5/3**	**Yes**	**64†**	**0.029/0.013**	**3/4**	**Yes**	**99#**	**0.022/0.025**	**3/3**	**Yes**
30	0.060/0.075	1/1	No	65	0.060/0.060	0/1	No	**100‡**	**0.022/0.029**	**3/3**	**Yes**
31*	**0.013/0.029**	**4/5**	**Yes**	**66§**	**0.025/0.029**	**2/4**	**Yes**	101	0.091/0.060	0/0	No

DS 10D (n = 105)											
32	0.091/0.060	1/0	No	67	0.091/0.060	0/0	No	**102**‡	**0.022/0.019**	**3/3**	**Yes**
33†	**0.025/0.025**	**4/3**	**Yes**	**68**§	**0.022/0.019**	**3/3**	**Yes**	103	0.122/0.060	0/1	No
34	0.060/0.060	0/1	No	69	0.122/0.060	0/1	No	104	0.091/0.091	0/0	No
35	0.122/0.091	0/0	No	70	0.091/0.091	0/0	No	**105**‡	**0.029/0.025**	**3/3**	**Yes**

DS 10D=desiccation stress for 10 days, TV=Tear Volume, CIS=Corneal Irregularity Score. R=Right, L=Left. * Indicates inclusion in the PBS 10D group (n = 19). † Indicates inclusion in the CDECM 10D group (n = 19). § Indicates inclusion in the DS 10D group (n = 19). # Indicates inclusion in the PBS 5D group (n = 3). ‡ Indicates inclusion in the CDECM 5D group (n = 3).

for 10 days after the removal of the DS). Ten days after the initiation of the experimental dry eye condition, the DS was removed by discontinuing the scopolamine injections and placing the mice in an environment with normal humidity and temperature. The mice were euthanized after 5 or 10 days of treatment.

Measurement of tear production: Tear production was measured using phenol red–impregnated cotton threads (Zone-Quick; Oasis, Glendora, CA), as previously described [41]. Briefly, the threads were held with jeweler's forceps and placed in the lateral canthus for 20 s. The tear volumes were expressed as the millimeters of wet thread that had been turned red by tears as measured under a microscope (SZX7; Olympus Corp., Tokyo, Japan). The tear fluid uptake was measured in millimeters and compared to a standard curve that was prepared from cotton threads with known uptake volumes of a stock basic solution (1,500 ml of 0.9% saline and 5 ml of 5 N NaOH) over 20 s and was within the range that would be expected for mouse tears [42,43]. The tear production measurements were obtained 2 h after the last scopolamine injection in the DS 10D group and 1 h after the last instillation in the PBS and CDECM groups [40]. The tear production was measured in both eyes, and the average value of both eyes was analyzed.

Evaluation of corneal smoothness: The corneal smoothness of the PBS and CDECM groups was assessed in three different sets of experiments. The reflected images of the white ring of a fiber optic ring illuminator of a stereoscopic zoom microscope were obtained immediately after euthanasia. The corneal smoothness was obtained 2 h after the last scopolamine injection in the DS 10D group and 1 h after the last instillation in the treatment groups (PBS and CDECM groups) [40]. The corneal smoothness was assessed by two blinded observers who graded the distortion of the white ring as a reflection off the corneal epithelium in the digital images, as previously described [15]. The corneal smoothness was measured in both eyes, and the average value of both eyes was measured. The corneal irregularity severity scores were calculated using a six-point scale that was based on the number of distorted quarters in the reflected ring and were graded as follows: 0, no distortion; 1, distortion in one quarter; 2, distortion in two quarters; 3, distortion in three quarters; 4, distortion in all four quarters; and 5, distortion so severe that no section of the ring was recognized.

Histology: The eyes and adnexa of each group were surgically excised, fixed in 10% formalin, and embedded in paraffin or optimal cutting temperature (OCT) compound (Tissue-Tek, Sakura Fine Technical Co., Ltd., Tokyo, Japan). Six-micrometer sections were stained with hematoxylin and eosin (H&E) and periodic acid Schiff (PAS). The eyes of each groups were assessed in an area of 0.1 mm^2 in the regions of the cornea or inferior fornices of the conjunctiva. The numbers of detached corneal epithelial cells and conjunctival goblet cells were measured by averaging the data from three nonconsecutive cross-section slides for each mouse and three or four mice per group. The sections from each group were examined and imaged using a Virtual Microscope (NanoZoomer 2.0 RS, Hamamatsu, Japan).

RNA isolation and quantitative real-time PCR: The mRNA expression patterns of the cornea and conjunctiva were quantified using quantitative real-time (qRT)-PCR analysis with the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA as an internal standard [16,17,19,20,44-46]. The total RNA from the corneas or conjunctivas were collected from each group (pooled 10 eyes per group for each experiment), and the RNA was isolated using a Purelink RNA Mini Kit (Life Technologies, Carlsbad, CA) according to the microcentrifuge pestle protocol of manufacturer's instructions. The RNA yield and quality were analyzed using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE). Next, 1 µg of the total RNA was reverse transcribed into cDNAs using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA). qRT-PCR analysis was employed using the SYBR Green PCR Core Reagents System (Applied Biosystems, Paisley, UK) and Applied Biosystems ViiA™ 7 Real-Time PCR System (Applied Biosystems). The primers (tumor necrosis factor-α [TNF-α], MMP-2, MMP-9, intercellular adhesion molecule-1 [ICAM-1], vascular cell adhesion molecule-1 [VCAM-1], and GAPDH) used in this study are provided in Table 3. The assays were performed in duplicate and repeated three times. The qRT-PCR results

Table 2. Classification after the desiccation stress.

Classification	DS 10D (n=19)	PBS 5D (n=3)	PBS 10D (n=19)	CDECM 5D (n=3)	CDECM 10D (n=19)
Tear production, µl (average value)	0.022	0.024	0.024	0.024	0.023
Irregularity score (average value)	3.316	3.167	3.447	3.000	3.447

were analyzed using the comparative threshold cycle method and normalized with GAPDH as an endogenous reference.

Western blotting analysis: The proteins from the corneas or conjunctivas from each group (pooled 20 eyes per group for each experiment) were extracted with cold lysis buffer (Intron Biotechnology, Seoul, Korea) [20,46]. Equal amounts of proteins in the cell lysates were separated on 8% sodium dodecyl sulfate (SDS)-polyacrylamide gels and then transferred electrophoretically onto a polyvinylidene difluoride (PVDF) membrane (Amersham, Buckinghamshire, UK). The membranes were blocked with 5% skimmed milk and then probed overnight with primary antibodies for TNF-α (1:1,000; Abcam, Cambridge, MA), MMP-9 (1:1,000; LifeSpan BioSciences, Inc., Seattle, WA), and ICAM-1 (1:1,000; Bioss Antibodies, Inc., Woburn, MA). After three washes with Tris-buffered saline containing 0.05% Tween-20 for 10 min each, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) or goat anti-mouse IgG (1:10,000; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. The chemiluminescent intensity was measured using the Fusion Fx image acquisition system (Vilber Lourmat, Torcy, France). β-Actin (1:5,000; Santa Cruz Biotechnology) was used as a loading control.

Statistical analyses: The data were analyzed with SPSS version 18.0 (SPSS, Chicago, IL) for Windows and expressed as mean ± standard deviation (SD). The differences between groups were analyzed using two-way analyses of variance (ANOVAs with Tukey's test), and statistical significance was defined as $p<0.05$.

RESULTS

Effects of CDECM on alterations in tear production: Instillation of PBS after the removal of the DS (PBS group) resulted in tear volumes that were still decreased at 3, 5, 7, and 10 days (Figure 1). After 10 days of DS (DS 10D), an 86% decrease in tear volume compared to day 0 was observed, and the PBS group exhibited a 2.2-fold increase after 10 days (0.056±0.019 µl) compared to the DS 10D group (0.025±0.004 µl; $p<0.05$). Instillation of CDECM after the removal of the DS (CDECM group) resulted in tear volumes that gradually improved to the control levels observed on day 0 at days 3, 5, 7, and 10. In addition, a significant 6.8-fold increase was observed in the CDECM group at 10 days (0.168±0.023 µl) compared to the DS 10D group (0.025±0.003 µl; $p<0.05$). The tear volume in the CDECM group was increased to 2.8-fold, 2.7-fold, and 3.0-fold at 5 (0.091±0.016 µl), 7 (0.110±0.016 µl), and 10 days (0.168±0.023 µl), respectively, compared to the PBS group ($p<0.05$).

Effects of CDECM on corneal surface irregularities: In the PBS group, the distortion of the white ring remained unchanged at 3, 5, 7, and 10 days. In contrast, the distortion of the white ring in the CDECM group improved to the control level observed on day 0 by days 7 and 10 (Figure 2A). The corneal irregularity score of the DS 10D group exhibited a sixfold increase compared to day 0, and the maximum increase observed in the PBS group was 7.5-fold at 10 days ($p<0.05$; Figure 2B). The irregularities in the CDECM group decreased to 60%, 70%, and 80% at 5, 7, and 10 days, respectively, compared to the PBS group ($p<0.05$). In addition, the corneal surface irregularities observed in the CDECM group gradually decreased to 75% of that observed in the DS 10D group ($p<0.05$).

Effects of CDECM on the detachment of corneal epithelial cells: The corneas of the NOD.B10.$H2^b$ mice were stained with H&E (Figure 3A). The number of detached corneal epithelial cells was increased by 7.5-fold and 2.0-fold in the PBS 10D and CDECM 10D groups, respectively, compared to the controls ($p<0.05$). The number of detached corneal epithelial cells was increased by eightfold in the DS 10D group compared to controls ($p<0.05$; Figure 3B). In contrast, the number of detached corneal epithelial cells observed in the PBS 10D group was decreased by 6.25% compared to the DS 10D group, but this number was reduced by 75% in the CDECM 10D group ($p<0.05$). In addition, the detachment of

Table 3. qRT-PCR primers.

Name	Primer sequence		Size (bp)
	Forward (5′-3′)	Reverse (5′-3′)	
TNF-α	CTGAACTTCGGGGTGATCGG	GGCTTGTCACTCGAATTTTGAGA	122
MMP-2	ACCTGAACACTTTCTATGGCTG	CTTCCGCATGGTCTCGATG	140
MMP-9	GGACCCGAAGCGGACATTG	CGTCGTCGAAATGGGCATCT	139
ICAM-1	TGCCTCTGAAGCTCGGATATAC	TCTGTCGAACTCCTCAGTCAC	121
VCAM-1	AGTTGGGGATTCGGTTGTTCT	CCCCTCATTCCTTACCACCC	112
GAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA	123

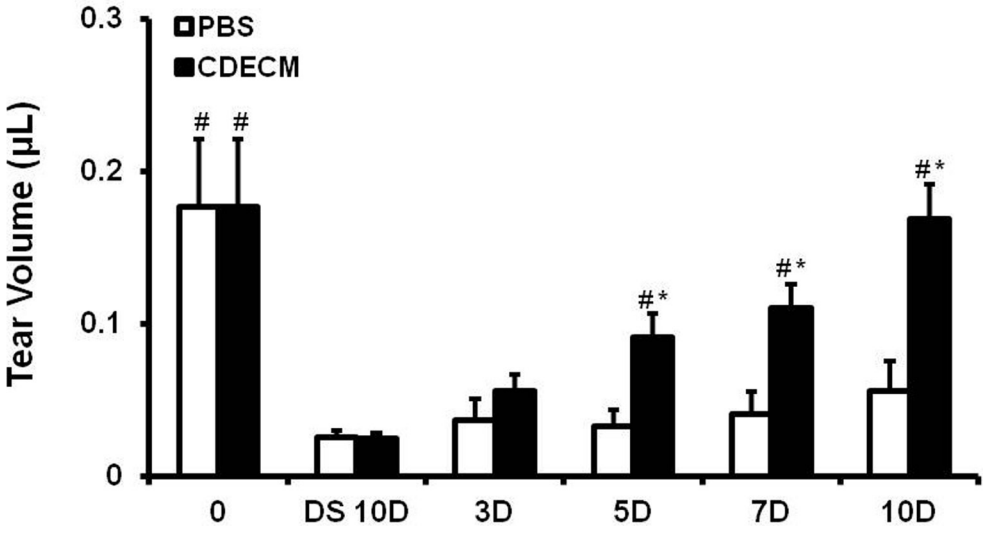

Figure 1. Effects of CDECM on tear production. Changes in the tear volumes of the experimental PBS- and chondrocyte-derived extracellular matrix (CDECM)-instilled NOD.B10.*H2*^{b} mice after the elimination of desiccation stress (DS). PBS or CDECM was instilled five times per day for the indicated number of days, and the tear volumes of the NOD.B10.*H2*^{b} mice were measured after 3, 5, 7, and 10 days of instillation. The quantitative data are presented as mean ± standard deviation (SD). *p<0.05 versus the value in the PBS group. #p<0.05 versus the corresponding value in the DS 10 day (10D) group.

corneal epithelial cells decreased to 73.3% in the CDECM group compared to the PBS group (p<0.05).

Effects of CDECM on the conjunctival goblet cells: The conjunctival goblet cells were observed following PBS and CDECM instillation after DS (Figure 4A). The number of conjunctival goblet cells decreased by 33.3% in the PBS 10D group compared to the controls, but increased by 1.1-fold in the CDECM 10D group (p<0.05). The number of conjunctival goblet cells decreased by 54.8% in the DS 10D group compared to the controls (p<0.05). In addition, the PBS 5D group exhibited a similar number of cells and recovered by 1.3-fold in the CDECM 5D group compared to the DS 10D group (p<0.05). The densities of the conjunctival goblet cells recovered by 1.4-fold in the PBS 10D group and by 2.3-fold in the CDECM 10D group compared to the DS 10D group (p<0.05). However, the number of goblet cells in the CDECM group increased 1.3-fold and 1.6-fold at 5 and 10 days, respectively, compared to the PBS group (p<0.05; Figure 4B). Thus, the PBS 5D and CDECM 5D groups were not significantly different, and a superior recovery of conjunctival goblet cells was observed in the CDECM 10D group compared to the PBS 10D group.

Anti-inflammatory effects of CDECM in the dry eye mouse model: The mRNA expression of TNF-α, MMP-2, MMP-9, ICAM-1, and VCAM-1 in the corneas (Figure 5A) and conjunctivas (Figure 5B) were assayed using qRT-PCR. In the corneas, the TNF-α (3.3-fold), MMP-2 (2.6-fold), MMP-9 (4.8-fold), ICAM-1 (6.2-fold), and VCAM-1 (5.6-fold) mRNAs were increased in the DS 10D group (p<0.05). The PBS group exhibited decreases of 55.2% for TNF-α and 13.2% for ICAM-1 compared to the DS 10D group (p<0.05). The CDECM group exhibited decreases of 72.4% for TNF-α, 16.2% for MMP-2, 76.2% for MMP-9, 83.3% for ICAM-1, and 69.2% for VCAM-1 compared to the DS 10D group (p<0.05). Moreover, the CDECM group was decreased by 38.4% for TNF-α, 37.5% for MMP-2, 80.3% for MMP-9, 86.2% for ICAM-1, and 64.5% for VCAM-1 compared to the PBS group (p<0.05; Figure 5A). In the conjunctivas, the TNF-α (8.5-fold), MMP-2 (3.4-fold), MMP-9 (3.3-fold), ICAM-1 (9.7-fold), and VCAM-1 (3.5-fold) mRNAs were increased in the DS 10D group (p<0.05). The PBS group exhibited decreases of 77.4% for TNF-α, 64.7% for MMP-9, and 70.2% for ICAM-1 compared to the DS 10D group (p<0.05). The CDECM group exhibited decreases of 94.3% for TNF-α, 83.4% for MMP-2, 89.3% for MMP-9, 82.8% for ICAM-1, and 68.7% for VCAM-1 compared to the DS 10D group (p<0.05). Moreover, the CDECM group was decreased by 74.8% for TNF-α, 92.3% for MMP-2, 69.7% for MMP-9, 42.5% for ICAM-1, and 73.7% for VCAM-1 compared to the PBS group (p<0.05; Figure 5B).

The protein expression levels of TNF-α (23–25 kDa), MMP-2 (72 kDa), MMP-9 (92 kDa), ICAM-1 (60 kDa), and VCAM-1 (81 kDa) in the corneas and conjunctivas were determined using western blotting analysis (Figure 6). The levels of the TNF-α and MMP-9 proteins were increased in the DS 10D group (1.5-fold and 2.8-fold, respectively) compared to the control, and the corneas of the CDECM group exhibited decreases in these proteins (63.5% and 27.1%, respectively) compared to the DS 10D group (p<0.05;

Figure 6A). In addition, the CDECM group exhibited the decreases in the TNF-α and MMP-9 proteins (39.8% and 31.4%, respectively) compared to the PBS group ($p<0.05$). The levels of the TNF-α and ICAM-1 proteins were increased in the conjunctivas of the DS 10D group (1.9-fold and 1.9-fold, respectively) compared to the control, and the conjunctivas of the CDECM group exhibited decreases in these proteins (38.8% and 55.4%, respectively) compared to the DS 10D group ($p<0.05$; Figure 6B). In addition, the CDECM group (44.2% and 27.9%) was exhibited decreases in the TNF-α and ICAM-1 proteins compared to the PBS group ($p<0.05$). No significant changes were observed for the MMP-2, ICAM-1, and VCAM-1 proteins in the corneas and the MMP-2, MMP-9, and VCAM-1 proteins in the conjunctivas (data not shown).

DISCUSSION

In the present study, we investigated the effects of CDECM on the cornea and conjunctiva in an experimental model of dry eye, in which PBS or CDECM was instilled following the removal of DS. In the CDECM group, the tear volumes and corneal smoothness scores were improved 10 days after CDECM instillation, and the goblet cell density in the conjunctiva, the numbers of detached epithelial cells in the cornea, and the expression of inflammatory factors had returned to the levels observed on day 0 by day 10. In contrast, in the PBS group, the tear volumes, corneal smoothness scores, conjunctival goblet cell densities, and number of detached corneal epithelial cells failed to recover to the day 0 levels over a period of 10 days after instillation.

Yoon et al. observed the tear production of C57BL/6 and NOD.B10.*H2*^{b} mice for 10 to 28 days after the removal of

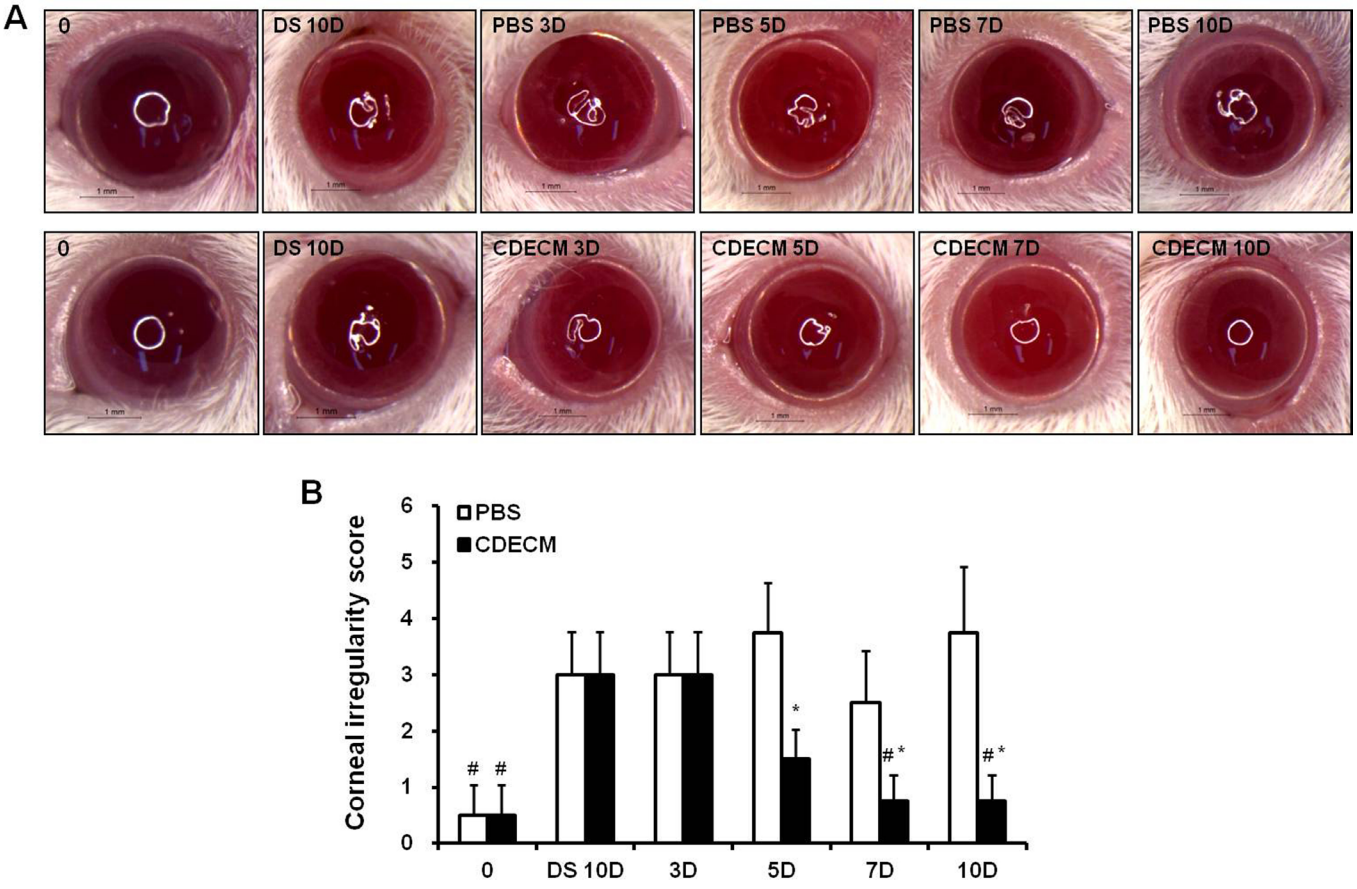

Figure 2. Effects of CDECM on the corneal surface irregularities. **A**: The eyes of the PBS and chondrocyte-derived extracellular matrix (CDECM) groups were imaged using a microscope after the removal of desiccation stress (DS). PBS or CDECM was instilled five times per day for the indicated number of days, and the corneal smoothness of the NOD.B10.*H2*^{b} mice was measured 3, 5, 7, and 10 days of instillation. Scale bar=1 mm. **B**: Changes in the corneal irregularity scores in the experimental NOD.B10.*H2*^{b} mice after the removal of DS in the PBS and CDECM groups. The quantitative data are presented as mean ± standard deviation (SD). *$p<0.05$ versus the value in the PBS group. #$p<0.05$ versus the corresponding value in the DS 10 day (10D) group.

DS [39]. The C57BL/6 mice recovered after 3 days but the NOD.B10.*H2*^{b} mice did not recover. In the present study, we examined the effects of the instillation of PBS and CDECM on tear production for 10 days after the removal of DS in two groups of NOD.B10.*H2*^{b} mice (Figure 1). We found that the CDECM group exhibited superior recovery in tear production compared to the PBS group. These results suggest that the instillation of CDECM ameliorated the decreased tear production in this dry eye model.

Corneal epithelial cells, which are damaged in dry eye conditions, have important roles that include the maintenance of the tear film and the permeability of the ocular surface. Using the same animal model, De Paiva et al. observed that the corneal smoothness scores and the numbers of desquamating apical corneal epithelia cells were improved using anti-inflammatory agents [15]. The authors reported that the corneal irregularity scores and the numbers of desquamating apical corneal epithelia cells decreased in animals treated with methylprednisolone and doxycycline compared to those in the DS group. Similarly, we demonstrated that instillation of CDECM for 10 days resulted in a significant improvement in DS, as determined by the corneal irregularity scores (Figure 2). In particular, significant differences in the corneal irregularity scores were observed at 5, 7, and 10 days in the CDECM group compared to the PBS group. In addition, the

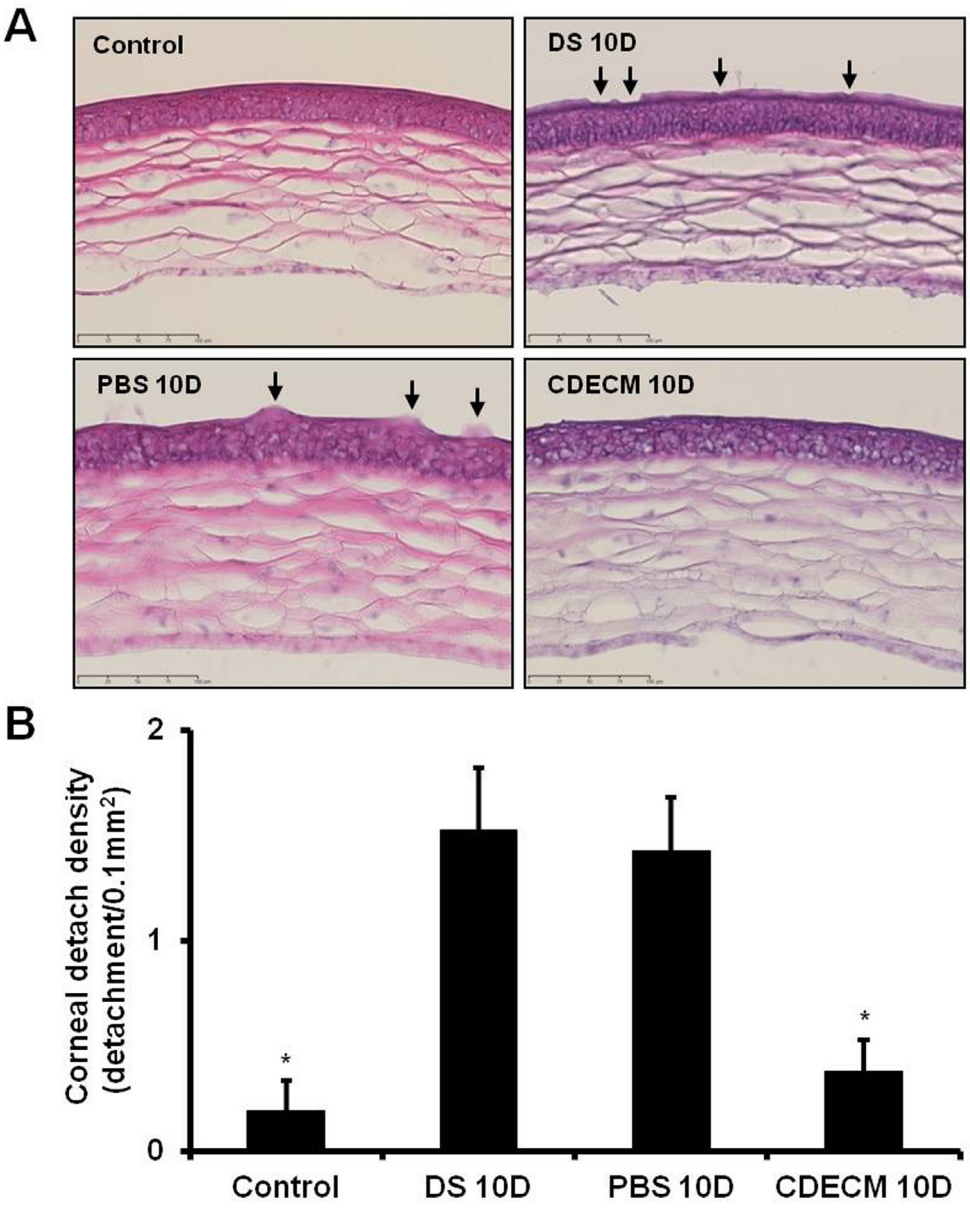

Figure 3. Effect of CDECM on the detachment of corneal epithelial cells. Stained corneas from the experimental NOD.B10.*H2*^{b} mice. **A**: Hematoxylin and eosin (H&E) staining: The corneas of the NOD.B10.*H2*^{b} mice were stained 10 days (10D) after the instillation of PBS or chondrocyte-derived extracellular matrix (CDECM) five times per day for 10D. The arrows indicate the detached corneal epithelial cells. Scale bar=100 µm. **B**: The quantitative data are presented as mean ± standard deviation (SD). *p<0.05 versus the value in the control. #p<0.05 versus the corresponding value in the DS 10D group. DS 10D, desiccation stress for 10 days; PBS 10D, PBS group after 10 days of instillation; CDECM 10D, CDECM group after 10 days of instillation.

number of detached corneal epithelial cells was decreased in the CDECM group compared to the DS group (Figure 3). Thus, CDECM improved the effect of corneal smoothness and the detachment of corneal epithelial cells. Conjunctival goblet cells are known to protect the cornea by secreting the gel-forming mucin MUC5AC and stabilizing the tear film. The loss of conjunctival goblet cells is a hallmark of dry eye [47,48]. Yüksel et al. previously suggested that the treatment of dry eye patients with cyclosporine A for 6 months could increase the density of goblet cells [21]. In addition, we found that the density of conjunctival goblet cells in the CDECM group recovered to a level similar to that of the controls and was increased compared to the DS 10D group (Figure 4). The secretion of mucin represents an important role for goblet cells in dry eye, and the instillation of CDECM increased the numbers of goblet cells.

In recent research, a variety of treatments have been investigated in dry eye, such as steroids, antibiotics, and omega-3 fatty acids [16,17,19,20,44-46]. Methylprednisolone and the antibiotic doxycycline were found to suppress the expression of MMP-9, interleukin-1α (IL-1α), IL-1β, and TNF-α mRNAs in the corneal epithelium in an experimental model of dry eye [16]. Another study demonstrated that doxycycline decreased the mRNA and protein levels of MMP-1, -13, and -10; the production of MMP-9; and the activity and expression of IL-1 in human corneal epithelial cells [17,20,44]. Furthermore, Sadrai et al. reported that azithromycin significantly reduced the expression of IL-1β, TNF-α, and

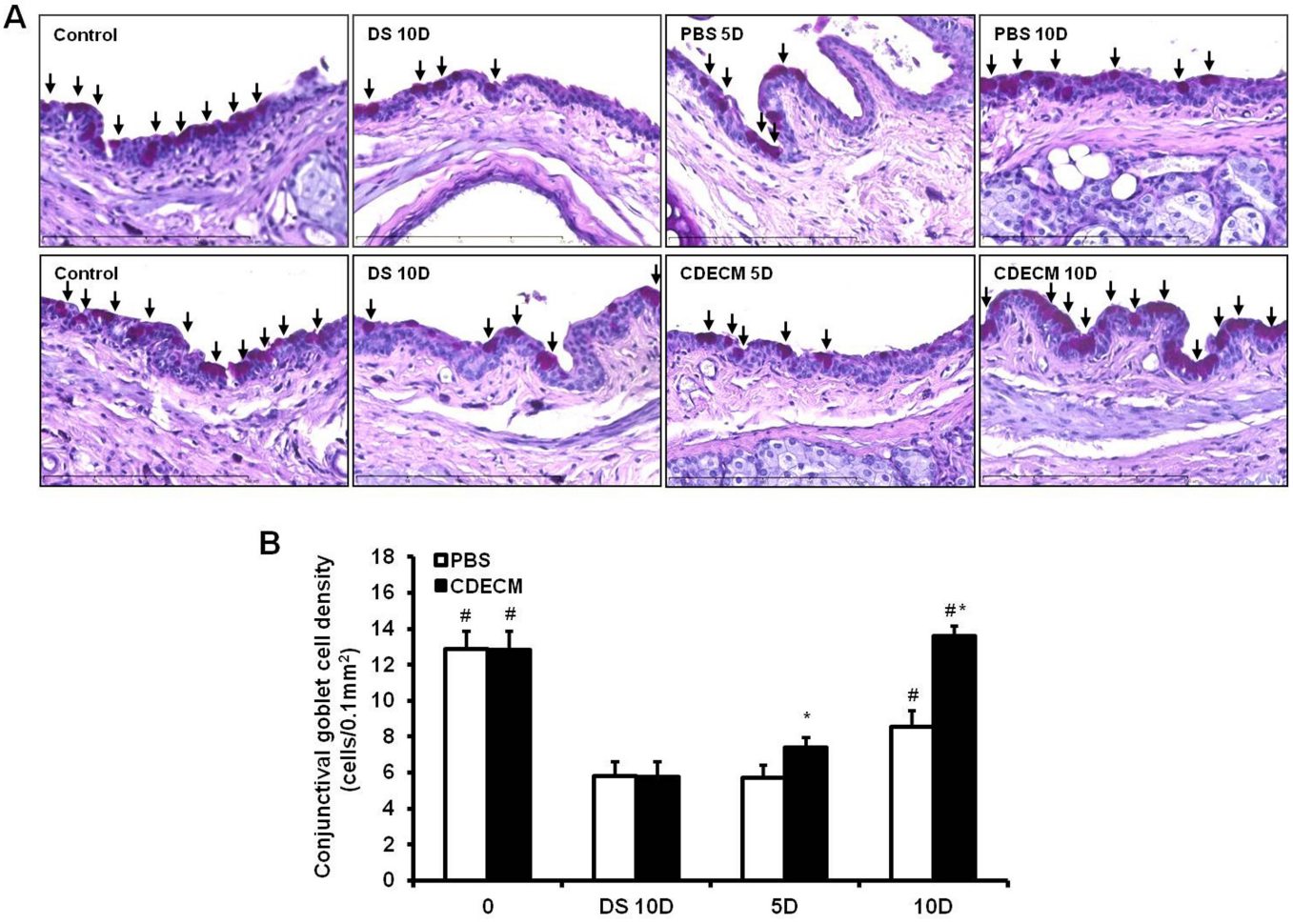

Figure 4. Effect of CDECM on the conjunctival goblet cell densities. **A**: The conjunctivas of the inferior fornices from the PBS and chondrocyte-derived extracellular matrix (CDECM) groups were stained with periodic acid Schiff (PAS). The arrows indicate the conjunctival goblet cells. Scale bar=200 μm. **B**: The quantitative data are presented as mean ± standard deviation (SD) *p<0.05 versus the value in the PBS group. #p<0.05 versus the corresponding value in the DS 10D group. DS 10D, desiccation stress for 10 days; PBS 5D, PBS group after 5 days of instillation; PBS 10D, PBS group after 10 days of instillation; CDECM 5D, CDECM group after 5 days of instillation; CDECM 10D, CDECM group after 10 days of instillation.

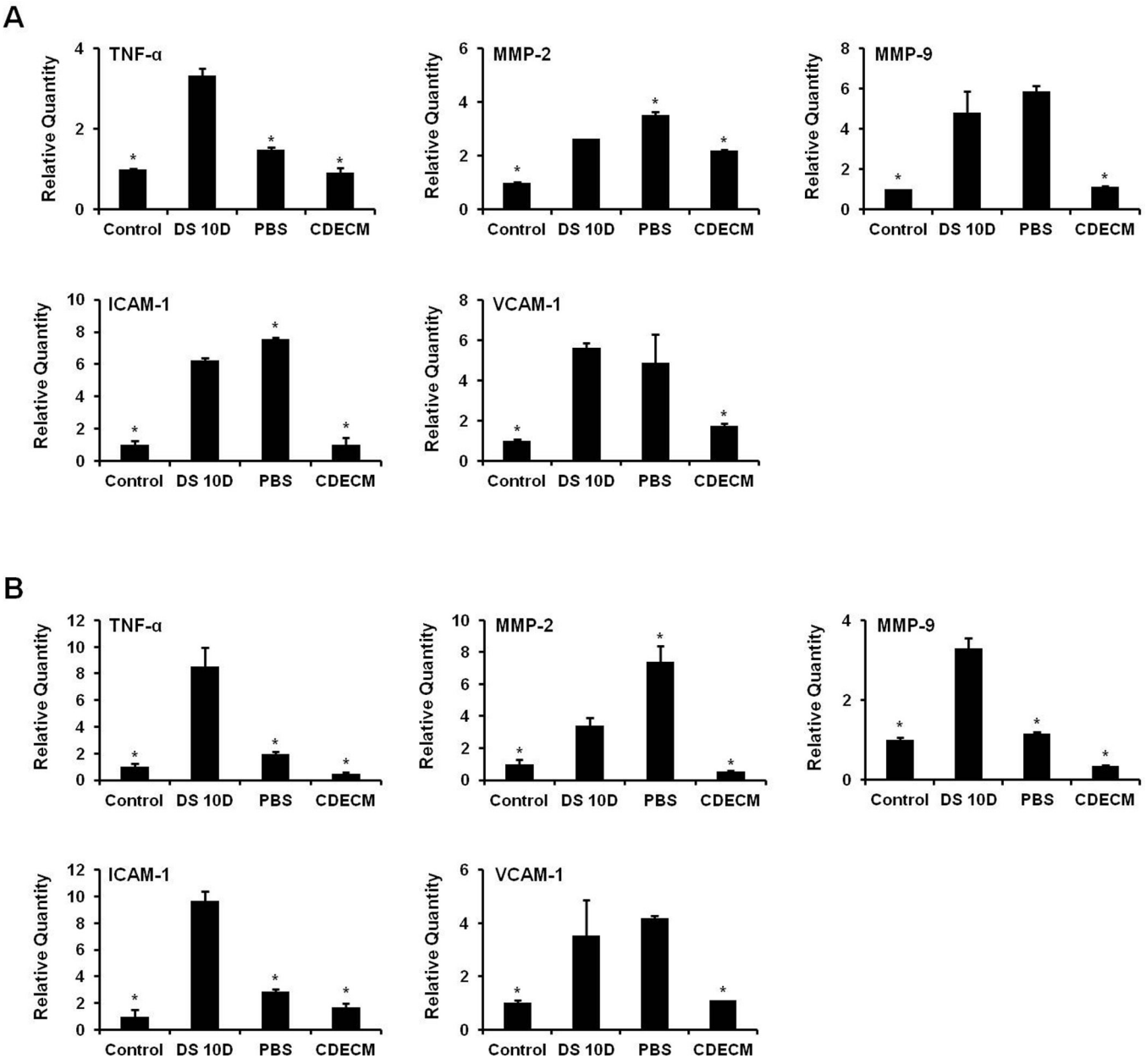

Figure 5. Effects of CDECM on the mRNA expression patterns of inflammatory factors. **A**: The mRNA expression in the corneas. **B**: The mRNA expression in the conjunctivas. Quantitative real-time (qRT)-PCR assessing the expression of the tumor necrosis factor (TNF-α), matrix metalloproteinase-2 (MMP-2), MMP-9, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) mRNAs in the corneas or conjunctivas of the NOD.B10.H2^{b} mice after the removal of desiccation stress and after 10 days of instillation of PBS or chondrocyte-derived extracellular matrix (CDECM) five times per day. The quantitative data are presented as mean ± standard deviation (SD). *p<0.05 versus the value in the DS 10D group. DS 10D, desiccation stress for 10 days; PBS, PBS group after 10 days of instillation; CDECM, CDECM group after 10 days of instillation.

ICAM-1 mRNAs in the cornea in a murine model of corneal inflammation [19]. Moreover, one of the omega-3 fatty acids, α-linolenic acid (ALA) has been proven to reduce the expression of corneal IL-1α and TNF-α and conjunctival TNF-α in the central cornea in an experimental dry eye mouse model [18]. As shown in Figure 5, the mRNA expression of inflammatory factors (i.e., TNF-α, MMP-2, MMP-9, ICAM-1, and VCAM-1) in the corneas and conjunctivas was significantly reduced in the CDECM group compared to the PBS group in our experimental dry eye mouse model. Surprisingly, we

found that CDECM reduced the levels of the TNF-α and MMP-9 proteins in the corneas and the TNF-α and ICAM-1 proteins in the conjunctivas (Figure 6). Thus, we suggest that the instillation of CDECM suppressed the expression of inflammatory factors in dry eye similar to those reported for steroids, antibiotics, and omega-3 fatty acids. CDECM had been proven to suppress vessel invasion, angiogenesis, neovascularization, adhesion, and proliferation in vivo and in vitro [31-37]. Therefore, CDECM will be considered as a treatment option for ocular surface inflammatory diseases.

In conclusion, our study suggests that CDECM promoted tear production, improved corneal surface smoothness, and aided in the recovery of conjunctival goblet cells in an experimental mouse model of dry eye. Furthermore, CDECM suppressed the detachment of corneal epithelial cells and reduced the expression of inflammatory factors. Therefore, CDECM is expected to have beneficial effects in the treatment of dry eye by improving the conditions on the ocular surface.

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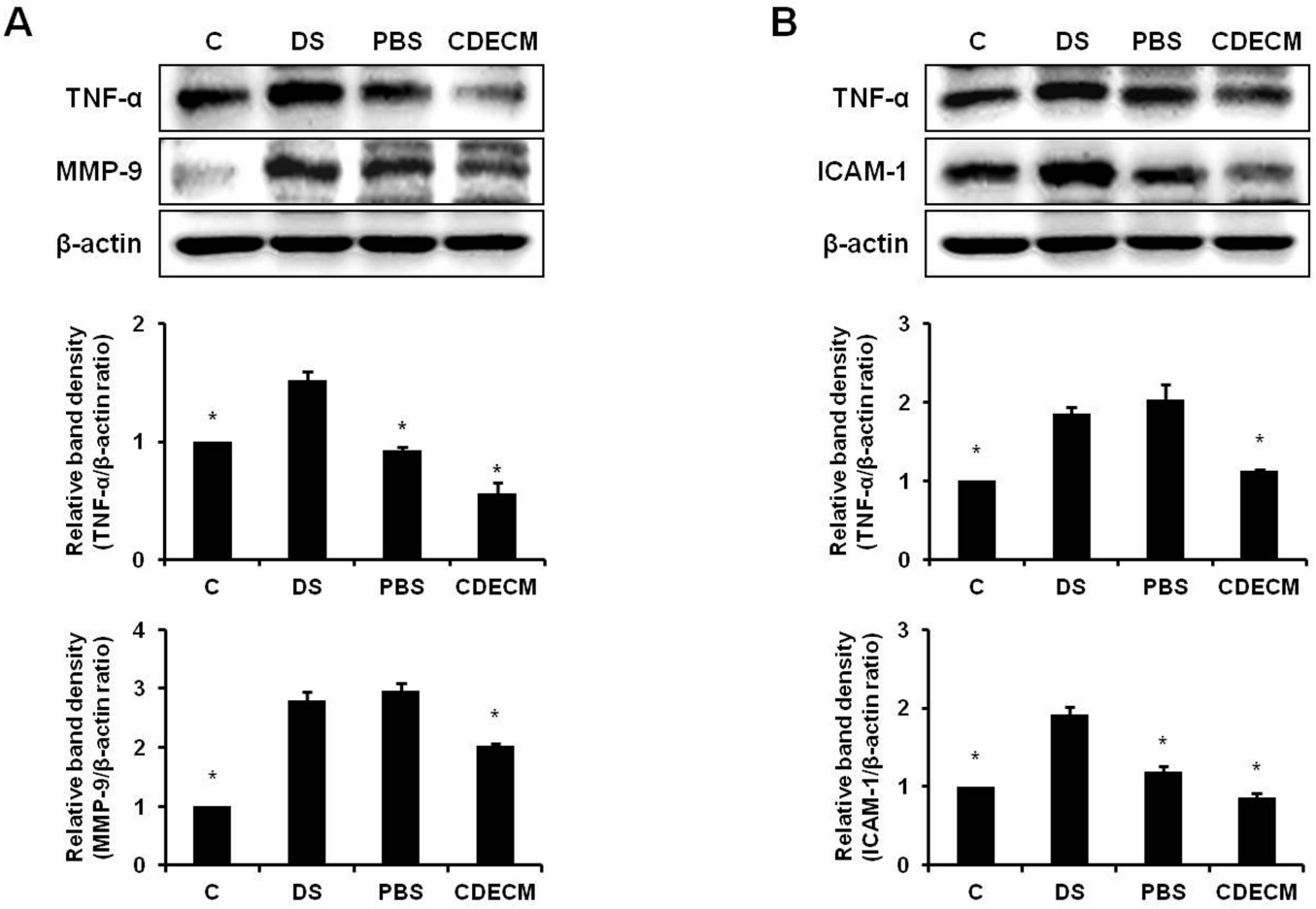

Figure 6. Effects of CDECM on the protein expression of inflammatory factors. **A**: The expression of the tumor necrosis factor-α (TNF-α) and matrix metalloproteinase-9 (MMP-9) proteins in the corneas. **B**: The expression of the TNF-α and intercellular adhesion molecule-1 (ICAM-1) proteins in the conjunctivas. Western blotting analyses determined the protein expression of inflammatory factors in the corneas or conjunctivas of the NOD.B10.H2^{b} mice after the removal of desiccation stress and after 10 days of instillation of PBS or chondrocyte-derived extracellular matrix (CDECM) five times per day. The quantitative data are presented as mean ± standard deviation (SD). *p<0.05 versus the value in the DS 10D group. C, Control; DS, desiccation stress for 10 days; PBS, PBS group after 10 days of instillation; CDECM, CDECM group after 10 days of instillation.

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