

Anti-Inflammatory and Antioxidative Effects of *Camellia japonica* on Human Corneal Epithelial Cells and Experimental Dry Eye: In Vivo and In Vitro Study

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PURPOSE. To analyze the anti-inflammatory and antioxidative effects of *Camellia japonica* (CJ) on human corneal epithelial (HCE) cells and its therapeutic effects in a mouse model of experimental dry eye (EDE).

METHODS. *Camellia japonica* extracts of varying concentrations (0.001%, 0.01%, and 0.1%) were used to treat HCE cells. Dichlorofluorescein diacetate (DCF-DA) and dihydroethidium (DHE) assays were performed. The production of peroxiredoxin (PRX) 1-6 and manganese-dependent superoxide dismutase (MnSOD) in HCE cells was assessed using Western blot analysis. Furthermore, eye drops containing 0.001%, 0.01%, or 0.1% CJ extract or a balanced salt solution (BSS) were applied to the EDE. Clinical parameters were measured 7 days after treatment. The levels of inflammatory markers and intracellular reactive oxygen species (ROS) were measured.

RESULTS. Treatment with 0.01% and 0.1% CJ extracts decreased apoptosis in HCE cells. In addition, band intensities of PRX 1, 4, and 5, as well as MnSOD, after hydrogen peroxide (H₂O₂) treatment showed a significant improvement after pretreatment with 0.01% and 0.1% CJ extracts. Mice treated with 0.1% CJ extract showed significantly improved clinical parameters when compared to those of the EDE control and BSS groups. A significant decrease in the levels of inflammatory markers and intracellular ROS was observed in the 0.01% and 0.1% CJ extract groups.

CONCLUSIONS. *Camellia japonica* extracts promoted antioxidative protein expression and suppressed apoptosis in HCE cells. Furthermore, CJ extracts improved clinical signs of dry eye and reduced oxidative stress and the expression of inflammatory markers, suggesting that eye drops containing CJ extract could be used as an adjunctive treatment for dry eye.

Keywords: apoptosis, corneal epithelial cells, *Camellia japonica*, dry eye, inflammation, oxidative stress

The Theaceae plant, *Camellia japonica* (CJ) is a broad-leaved evergreen woody species that has been widely cultivated as an ornamental plant in the Asian region.^{1,2} *Camellia japonica* oil long has been used as a cosmetic protectant for healthy skin and hair, and often is used as a soothing agent.² In addition, the flower petals and buds of CJ have been used in the treatment of bleeding and blood stagnation, as well as in oriental traditional medicine owing to its anti-inflammatory properties.^{3,4} Recent studies suggest that CJ has various biological functions, including antibacterial and antiallergic activity, inhibition of human immunodeficiency virus type 1 protease, inhibition of Epstein-Barr virus, antimetastatic activity, and inhibition of human type 1 procollagen production.⁴⁻¹¹ In addition, CJ leaf extract is known to exhibit potent antioxidant activity because of high content of

polyphenolic compounds.^{8,12} *Camellia japonica* oil exerts its anti-inflammatory effect by downregulating the expression of inducible nitric oxide synthase (iNOS) and cyclo-oxygenase-2 (COX-2) genes through inhibition of nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1) signaling.² The following are the biologically active components of CJ leaves: (–)-epicatechin and (+)-catechin; the anti-inflammatory and antiallergic substances okicamelliaside, quercetin-3-β-D-glucoside, and eugenol; and the diphenylpicrylhydrazyl (DPPH) radical scavenging compounds camellianoside, rutin, hyperoside, and isoquercitrin.^{8,11,13,14} Studies have been conducted on the biologic effects of CJ on skin in vitro and in vivo to evaluate its antiaging and anti-inflammatory properties.¹⁰ However, few studies have investigated the effectiveness of CJ in treating eye diseases.



Dry eye disease (DED) is a common ocular surface disorder described as a derangement of the tear film caused by reduced tear production, poor tear quality, or excessive tear evaporation.¹⁵ The pathogenesis of DED is not entirely understood; however, it has been recognized that inflammation has a key role in the development of DED. Phosphorylation of the stress-activated mitogen activated protein kinases (MAPKs) p38 and c-Jun N-terminal kinase (JNK), followed by activation of transcription factors, such as AP-1 and NF- κ B, results in increased levels of proinflammatory cytokines, such as IL-1 β , TNF- α , IL-8, and IL-6.^{16–20} Another contributor to ocular surface damage in DED is reactive oxygen species (ROS) overproduction and the resultant oxidative stress.²¹ A close relationship among lipid peroxidation-related membrane damage, protein oxidation, ROS production, and inflammation has been demonstrated in patients and animal models of DED.²² Antioxidants, such as oral sea buckthorn oil, green tea polyphenol epigallocatechin gallate, and topical selenoprotein P, have been used in animal models and patients with DED. These have proven to alleviate DED parameters, indicating that ROS have an important role in the pathogenesis of this disease.^{23–26}

Based on the potent anti-inflammatory and antioxidative properties of CJ, we focused on investigating the in vitro and in vivo effects of CJ leaf extracts using human corneal epithelial (HCE) cells and murine experimental dry eye (EDE) model.

METHODS

Preparation of CJ Leaf Extracts

Fresh CJ leaves were collected from Jangseong province in South Korea in May 2014. The collected leaves were washed with distilled water and then placed in a dry oven set at 40°C for 10 days, until their moisture content was reduced to less than 5%. A pin-type mill then was used to grind the leaves to a size of 0.5 mm. The samples were sieved through a 45- μ m mesh screen. Supercritical CO₂ extraction was done using the ISA-SCFE system (Ilshin Company, Daejeon, South Korea) at the Nano Bio Research Center in Jangseong province. Pure CO₂ was applied using a syringe pump. Each ground leaf, weighing 100 to 125 g, was placed in separate vessels, and extracted supercritically using CO₂ as the main extraction gas and C₂H₅OH as the co-solvent, under a pressure of 200 bar.^{27,28} Optimized operational parameters were extraction time, temperature, and pressure. The extraction conditions for each separation vessel were 40°C, 200 bar pressure, and flow rates of 60 ml/min for the CO₂ and 3 ml/min for the cosolvent. These conditions were maintained for 2 hours. The parameters were optimized using a pretest and were judged based on extraction efficiency and operational performance. The temperature and pressure were optimized using an experimental design because they are essential for extraction. The characteristics of resulting CJ extracts were yellow liquid with a density of 0.818 g/mL. A preliminary phytochemical investigation revealed the presence of quercetin, oxalic acid, sabinene, limonene, kaempferol, terpenes, and flavonoids in the extracts. After extraction, the liquids were stored in a clean vial at –20°C until use.

During the in vitro part of our experiment, we used phosphate buffer solution (PBS) to dilute CJ extract on the day of the experiments. During the in vivo part of our experiment, we used balanced salt solution (BSS) to dilute CJ extract on the day of the experiments. *Camellia japonica* extracts were diluted to concentrations of 0.001%, 0.01%, and 0.1% in both parts of the experiment.

Culture of HCE Cells and Cell Viability Assay

Epithelial adenovirus 12-SV40 hybrid-transformed HCE cells were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) at passage 28 (Cat No. CRL-11135). The cells were cultured at 37°C in a humidified incubator containing 5% CO₂, maintained in Dulbecco's modified Eagle's medium, and supplemented with human corneal growth supplement (Cascade Biologics, Portland, OR, USA), 100 U/mL penicillin, and 100 μ g/mL streptomycin.

Cell viability was determined using an EZ-Cytox assay kit (Daeil Lab Service, Seoul, South Korea) based on water-soluble tetrazolium salt (WST).²⁹ Briefly, the HCE cells (2×10^5 cells/well) were seeded in a 96-well plate and incubated for 24 hours to reach approximately 70% confluence. Subsequently, the cells were pretreated with either different concentrations of CJ extracts (0.001%, 0.01%, and 0.1% diluted in PBS) for 1 hour or PBS only as a negative control, followed by rinsing, and then reincubation for another 24 hours. Water-soluble tetrazolium salt reagent solution (10 μ L) then was added to each well, and the plate was reincubated for 3 hours in a CO₂ incubator. An ELx808 absorbance microplate reader (BioTek, Winooski, VT, USA) was used to measure the absorbance at 570 nm. Hydrogen peroxide (H₂O₂) (Fisher Scientific, Leicestershire, UK) was used as a positive control to assess the effect of the CJ extracts on cell viability after oxidative stress.³⁰ The cells were pretreated with CJ extracts at the indicated concentrations (0.001%, 0.01%, and 0.1%) for 1 hour before exposure to H₂O₂ (200 μ M). Exposure to H₂O₂ lasted for 30 minutes. Thereafter, cell viability was measured using the EZ-Cytox assay. *Camellia japonica* extract-pretreated and H₂O₂-treated cells were used for other in vitro analyses, and each assay was performed in triplicate.

Determination of Intracellular ROS

The level of intracellular ROS was determined using dichlorodihydrofluorescein diacetate (DCF-DA) and dihydroethidium (DHE) assay kits according to the manufacturer's protocol. Briefly, HCE cells were washed twice in PBS, and then 10 μ M chloromethyl-H₂DCF-DA (C6827; Life Technologies, Darmstadt, Germany) or 5 μ M DHE (D-23107; Life Technologies) was added to the culture medium. The cells were incubated for 20 minutes and then washed twice again in PBS. Fresh PBS then was added and the cells were observed using fluorescence microscopy (Eclipse TE-300; Nikon, Melville, NY, USA) with the following fluorescence excitation and emission settings: excitation between 460 and 500 nm and emission between 515 and 550 nm for chloromethyl-H₂DCF-DA, and excitation at 518 nm and emission at 605 nm for DHE. The exposure time remained the same for all samples in all experiments. The DCF-DA and DHE fluorescence intensities were quantified using ImageJ software (version 1.45; <http://imagej.nih.gov/ij/>), provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) and expressed as percent normalized to control.

Measurement of Change in Mitochondrial Membrane Potential

Mitochondria are one of the most important cytoplasmic organelles and supply most of the necessary energy for cellular activities and functions. To detect whether CJ extracts alter mitochondrial function in cells subjected to H₂O₂-induced oxidative stress, a well-characterized JC-1 probe was used to evaluate the loss of mitochondrial membrane potential.³¹

Camellia japonica extracts-pretreated and H₂O₂-treated cells were cultured on plates and incubated with 2 μM 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazolylcarbocyanine iodide (JC-1; Life technologies) for 30 minutes. For the subsequent flow cytometry analysis, the cells were washed with PBS and incubated with Accutase solution (Innovative Cell Technologies, San Diego, CA, USA). After 2 minutes of incubation, the plates were carefully tapped to detach the cells. The suspended cells then were washed twice with cold PBS and resuspended in cold PBS. Flow cytometry was used to quantify JC-1 fluorescence. A decrease in red fluorescence (F_{2,585}) accompanied by an increase in green fluorescence (F_{2,510}) indicated a lowered mitochondrial membrane potential. The percentage of red fluorescence relative to the total fluorescence (summation of red and green fluorescence) was calculated.

Protein Extraction and Western Blotting

Cells were lysed in RIPA buffer (#9806; Cell Signaling Technology, Danvers, MA, USA) containing a protease inhibitor cocktail (Sigma-Aldrich Corp., St. Louis, MO, USA) and phosphatase inhibitor cocktail I + II (Sigma-Aldrich Corp.). An NE-PER nuclear and cytoplasmic extraction kit (#78835; Thermo Fisher Scientific, San Jose, CA, USA) was used to extract nuclear proteins.

Each fractional protein was extracted according to the manufacturer's instructions. Protein levels were quantified using the Bradford procedure. Whole cell extracts (30 μg each) were separated using SDS-PAGE and transferred onto enhanced nitrocellulose membranes. The blots then were washed with 10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% Tween-20 (TBST), blocked with 5% skim milk for 1 hour, and incubated overnight at 4°C with primary antibodies at the dilutions recommended by the supplier. The membranes then were washed with TBST and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature. The bands for the peroxiredoxin (PRX) family, manganese-dependent superoxide dismutase (MnSOD), catalase (CAT), and COX-2 were visualized using EZ-Western Lumi Pico Western Blotting Detection Reagent (Daeil Lab Service) on a luminescent image analyzer (Image Quant LAS4000 mini; GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, United Kingdom). Numerical values were generated by dividing the density of each band by that of the β-actin band in the same sample.

RNA Extraction, Reverse Transcription, and Quantitative RT-PCR

Total RNA was extracted from the cells using TRIzol, which is a monophasic solution of phenol and guanidine isothiocyanate purchased from Invitrogen (Carlsbad, CA, USA). Reverse transcription was performed using 1 μg of RNA in an RT Premix reverse transcription system kit (AccuPower, Seoul, Korea) with oligo-dT18 primers, and 0.5 μL of the RT products was amplified with Power SYBR Green (Applied Biosystems, Warrington, UK). The primer sequences for RT-PCR amplification were as follows: PRX1; sense, 5'-AAAGCCACAGCTGTATGCC-3' and antisense, 5'-A AGCACCAATCACCTTGCCAG-3', Catalase (CAT); sense, 5'-TGATCGGGGATTCCAGATG-3' and antisense, 5'-CCGGATGCCA TAGTCAGGAT-3', β-actin; sense, 5'-AGGCCAGAGCAAGAGAG-3' and antisense, 5'-TCAACATGATCTGGGTCATC-3'. Polymerase chain reaction was performed for 30 cycles at 95°C for 15 seconds, 60°C for 60 seconds, and 72°C for 40 seconds. The results were analyzed using the comparative threshold cycle method and normalized using β-actin as an internal control.

Mouse Model of Dry Eye and Experimental Design

The animal research protocol was approved by the Chonnam National University Medical School Research Institutional Animal Care and Use Committee (CNU IACUC-H-2015-11). Maintenance of animals and all in vivo experiments were performed in accordance with the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research. Female C57BL/6 mice aged 6 to 8 weeks were used in the following experiments. We induced EDE by subcutaneous injection of 0.5 mg/0.2 mL scopolamine hydrobromide (Sigma-Aldrich Corp.) four times a day (8 AM, 11 AM, 2 PM, and 5 PM) with exposure to an air draft and 30% ambient humidity, as described previously.^{32,33} During the experiments animal behavior, and food and water intake were not restricted.

The mice were separated randomly into five groups, each containing five mice, with each group receiving a different topical treatment. The five groups were as follows: (1) EDE control, mice that received no eye drops; (2) vehicle control, EDE mice treated with BSS (Alcon, Fort Worth, TX, USA); (3) EDE mice treated with 0.001% CJ extract; (4) EDE mice treated with 0.01% CJ extract; and (5) EDE mice treated with 0.1% CJ extract. The CJ extracts were diluted in BSS for each treatment group. Eye drops (2 μL) were applied topically to both eyes of the mice three times a day (8 AM, 12 PM, and 5 PM), until they were euthanized. Clinical parameters, including tear volume, tear film break-up time (BUT), and corneal fluorescein staining scores were measured in that order for 7 days after treatment. The measurements were made 3 hours after the last scopolamine injection and eye drop application. The mice were immobilized by intraperitoneal injection of 1 mg pentobarbital during measurement of the clinical parameters. After measuring the clinical parameters, the mice were euthanized using an intraperitoneal overdose of pentobarbital, ensuring minimum suffering. A multiplex immunobead assay and measurement of conjunctival ROS production using a DCF-DA assay were performed after tissue harvesting. All experiments and analyses were repeated three times.

Tear Volume Measurements

Tear volume measurements were obtained using phenol red-impregnated cotton threads (Zone-Quick; Oasis, Glendora, CA, USA) as described previously.³³ The lower eyelid was pulled down slightly, and the tips of the threads were placed on the peripheral conjunctiva at the lateral canthus for 20 seconds. The thread turned red when it was wet by tears, and the tear volume, expressed in millimeters of red thread, was measured using a microscope (SMZ 1500; Nikon). Each eye was tested three times and the average length of red thread was recorded as the definitive value. The measured uptake of tears in micrometers was compared to a standard curve prepared using cotton threads with a known uptake volume of basic stock solution (1500 mL of 0.9% saline combined with 5 mL of 5M NaOH) over 20 seconds, with volumes in the range that would be expected in a mouse tear.

Evaluation of Tear Film Break-Up Time and Corneal Fluorescein Staining

The tear film BUT and corneal fluorescein staining score measurements were conducted as previously described.³⁴ Sodium fluorescein (1%, 1 μL volume) was instilled into the inferior conjunctival sac using a micropipette. After three blinks, the BUT was recorded in seconds using slit-lamp biomicroscopy (BQ-900; Haag-Streit, Bern, Switzerland) under cobalt blue light. At 90 seconds later, punctate staining on the

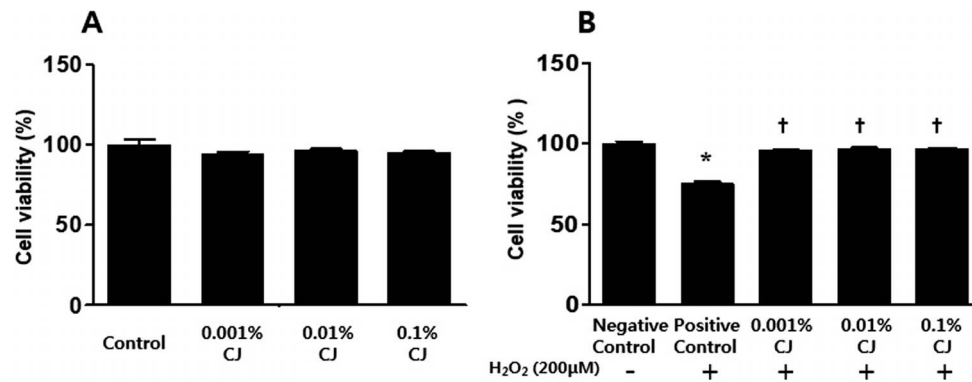


FIGURE 1. Effect of pretreatment with CJ extract on the viability of HCE cells. (A) The effect of CJ extract (0.001%, 0.01%, and 0.1% concentration) on the viability of HCE cells is shown. (B) The effect of CJ extract (0.001%, 0.01%, and 0.1% concentration) on HCE cells exposed to 200 μ M H₂O₂. The results are shown as a percentage relative to the control samples. * $P < 0.01$ compared to the control cells. † $P < 0.01$ compared to the cells exposed to H₂O₂.

corneal surface was measured in a masked fashion. Each cornea was divided into four quadrants that were scored individually. The intensity of corneal fluorescein staining was calculated using a 4-point scale: 0, absent; 1, superficial stippling micropunctate staining with <30 spots; 2, punctate staining with >30 spots, but no diffuse staining; 3, severe diffuse staining, but no positive plaque/patch; and 4, positive fluorescein plaque/patch. The scores of the four areas were summed to generate a final grade, which ranged from 0 to 16.

Multiplex Immunobead Assay for Inflammatory Cytokines and Chemokines

A multiplex immunobead assay (Luminex 200; Luminex Corp., Austin, TX, USA) was used to measure the concentrations of IFN- γ , IL-1 β , IL-6, TNF- α , IFN- γ -induced protein (IP)-10, and monokine induced by IFN- γ (MIG) in the conjunctiva, as previously described.³⁵ Briefly, the conjunctival tissues were collected (five eyes per group), pooled in a lysis buffer containing protease inhibitors, and incubated for 30 minutes. The cell extracts then were centrifuged at 14,000g at 4°C for 15 minutes, and the supernatants were stored at -70°C until use. The subsequent steps of the multiplex immunobead assay were performed within 2 days of storage. The total protein concentration of the supernatants was determined, and 25 μ L of each sample was pipetted into assay plate wells. The wells contained the appropriate cytokine and chemokine bead mixture (Millipore, Billerica, MA, USA) and mouse monoclonal antibodies specific for the mixture. After 2 hours, the supernatant was washed three times and a biotinylated secondary antibody mixture was applied for 1 hour, in the dark, at room temperature. The reactions were detected using a specialized analysis system (xPONENT, Austin, TX, USA) after addition of streptavidin-phycoerythrin. The concentrations of the tissue cytokines and chemokines present in the samples were calculated through comparison with standard curves of known concentrations of recombinant mouse cytokines and chemokines.

Measurement of Cellular ROS Level

The level of intracellular ROS was measured using a DCF-DA assay according to the manufacturer's protocol, and analyzed using flow cytometry. The conjunctiva of the mice (five eyes per group) were surgically harvested and placed in a 96-well plate containing 200 μ L of Krebs-Ringer bicarbonate buffer per well. The cells were incubated in the dark with 10 μ M DCF-DA (Molecular Probes, Eugene, OR, USA) for 30 minutes at 37°C. The plates then were read at an excitation wavelength of 480

nm and emission wavelength of 530 nm (FACSCalibur cytometer; BD Biosciences, San Jose, CA, USA). Data analysis was based on 10,000 detected events using flow and image cytometry analysis software. The results were expressed as the mean percentage increase of DCF-DA fluorescence over that of the control tissue (conjunctival tissue harvested from mice that had not been exposed to desiccant stress or topical treatment).

Statistical Analysis

SPSS version 18.0 (SPSS, Chicago, IL, USA) was used for statistical analyses. Results are presented as the mean \pm SD. The normal distribution of the data was verified using the Kolmogorov-Smirnov test. Statistical differences in tear volume, BUT, and corneal fluorescein staining among the groups were determined by 1-way ANOVA tests followed by Dunnett's post hoc tests (sphericity assumptions were evaluated with a Mauchly's test, and in the case of violation, the data were adjusted with an Epsilon Greenhouse-Geisser statistic). A Kruskal-Wallis test with Bonferroni post hoc analysis was used to compare cell viability, relative expression of mRNA, cytokine and chemokine levels, and DCF-DA value differences between the groups. A P value < 0.05 was considered statistically significant.

RESULTS

The Effect of CJ Extracts on H₂O₂-Induced Cytotoxicity in HCE Cells

As illustrated in Figure 1A, treatment with CJ extracts alone at the indicated concentrations (0.001%, 0.01%, and 0.1%) did not have any significant effect on the overall viability of HCE cells. This indicated that the concentrations used in this study were noncytotoxic. A significant decrease in overall viability of HCE cells exposed to 200 μ M H₂O₂ ($75.18 \pm 2.31\%$, $P < 0.01$ versus control) was observed (Fig. 1B). However, pretreatment with CJ extracts at the indicated concentrations significantly inhibited the H₂O₂-induced cytotoxicity (0.001% CJ extract, $95.70 \pm 1.23\%$; 0.01% CJ extract, $97.17 \pm 0.91\%$; and 0.1% CJ extract, $96.71 \pm 1.41\%$; all $P > 0.05$ versus negative control and all $P < 0.01$ versus H₂O₂ control).

Effect of CJ Extracts on ROS Level

Digital images of DCF fluorescence were observed using a fluorescence microscope system, and magnified images of the

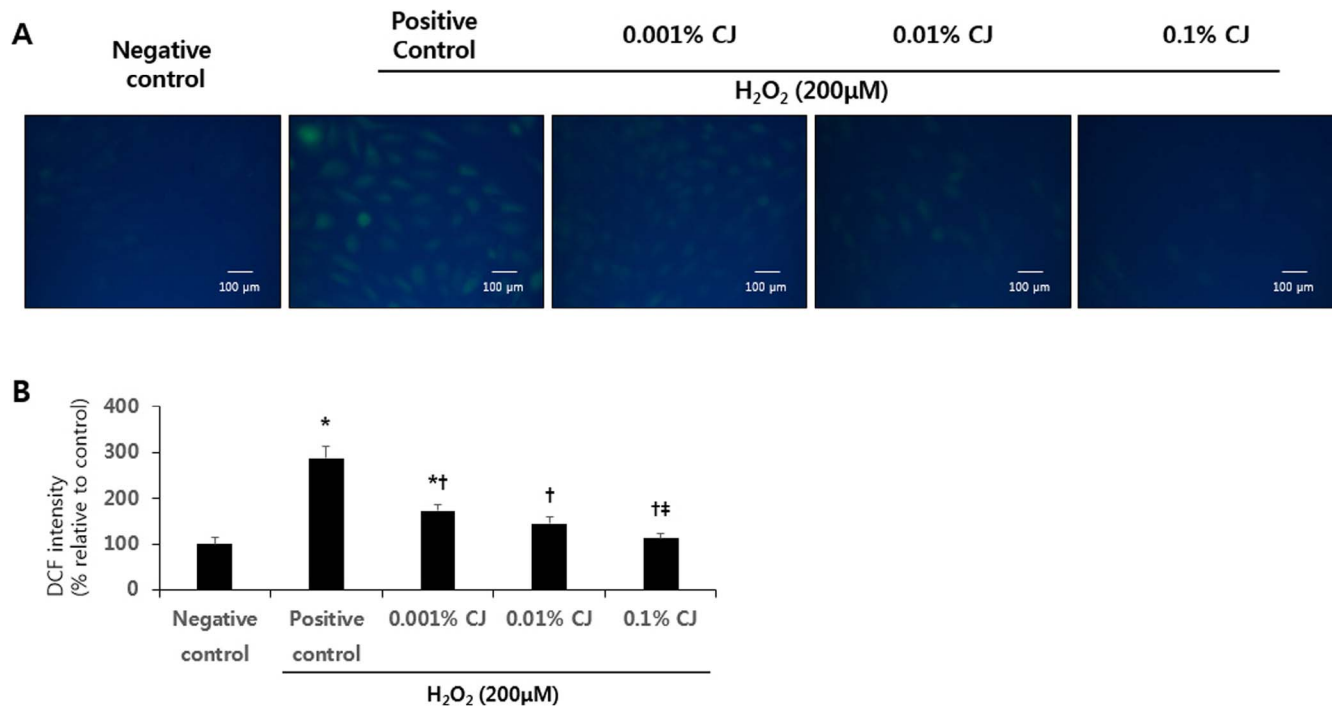


FIGURE 2. Staining with DCF-DA staining, and the subsequent confocal fluorescence microscopy observations in H₂O₂ (200 μM)-treated HCE cells with or without pretreatment with CJ extracts (0.001%, 0.01%, and 0.1% concentrations). **(A)** One representative image selected from three individual experiments is shown. **(B)** Relative fluorescence intensity (expressed as percent normalized to negative control) analysis is shown. **P* < 0.01 compared to the control cells. †*P* < 0.01 compared to the H₂O₂-exposed cells. ‡*P* < 0.05 compared to the 0.001% CJ extract pretreated cells.

representative DCF-DA and DHE staining as well as the analysis of relative fluorescence intensity, are illustrated in Figures 2 and 3. Treatment of HCE cells with H₂O₂ resulted in increased DCF-DA and DHE fluorescence (*P* < 0.01 versus negative control). However, pretreatment with CJ extracts before exposure to H₂O₂ significantly reduced DCF-DA fluorescence intensity in a concentration-dependent manner (0.001% CJ, *P* = 0.03 versus positive control; 0.01% CJ, *P* = 0.02 versus positive control; 0.1% CJ, *P* = 0.01 versus positive control and *P* = 0.03 versus 0.001% CJ). This tendency also was observed in the analysis of DHE fluorescence intensity (0.001% CJ, *P* = 0.02 versus positive control; 0.01% CJ, *P* < 0.01 versus positive control; 0.1% CJ, *P* < 0.01 versus positive control).

Effect of CJ Extracts on Mitochondrial Depolarization

Depolarization of mitochondrial membrane potential is implicated in ROS production and apoptosis.³⁶ Therefore, we measured the mitochondrial membrane potential of the HCE cells using JC-1 staining. A reduction in the ratio of red to total (red + green) fluorescence indicates a decrease in mitochondrial membrane potential. Hydrogen peroxide treatment of HCE cells resulted in a markedly reduced red fluorescence signal (*P* < 0.01), whereas pretreatment with CJ extract significantly increased the red fluorescence signal when compared to that of the H₂O₂ control in a concentration-dependent manner (all *P* < 0.01 versus H₂O₂ control; 0.01% CJ, *P* = 0.04 vs. 0.001% CJ; 0.1% CJ, *P* < 0.01 vs. 0.001% CJ). The 0.01% CJ and 0.1% CJ groups did not show a significant difference in the proportion of red fluorescence when compared to that of the naïve control (all *P* > 0.05; Figs. 4A, 4B).

Effect of CJ Extracts on Anti-Inflammatory and Antioxidative Markers

Proteins in the PRX family are induced by various oxidative stimuli and protect cells against oxidative radical damage caused by ROS.³⁷ Cyclo-oxygenase-2 is considered to be a proinflammatory enzyme because free radicals and prostaglandins are produced during its catalytic cycle.³⁸ Upregulation of COX-2 is a common feature of inflammation caused by oxidative stress.^{38–41} Manganese-dependent superoxide dismutase is an antioxidant enzyme and has a critical role in scavenging ROS.⁴⁰ As shown in Figure 5, H₂O₂ pretreatment dramatically reduced the levels of the PRX family proteins, MnSOD and CAT, and increased the level of COX-2. However, in general, the band intensity of the PRX family proteins (especially PRX 1, 4, and 5), MnSOD, and CAT significantly increased in a dose-dependent manner in HCE cells pretreated with CJ extract (especially with the 0.1% CJ extract). In addition, the band intensity of the COX-2 was significantly decreased in the 0.1% CJ extract group when compared to that in the H₂O₂ control and the 0.001% CJ extract group.

Effect of CJ Extracts on mRNA Expression of Antioxidant Enzymes

To further analyze the effects of CJ extracts during oxidative stress, we measured the expression of enzymes involved in antioxidant defenses. Exposure of HCE cells to H₂O₂ significantly decreased mRNA expression of PRX1 and CAT (*P* = 0.02 and 0.04, respectively) (Fig. 6). After pretreatment with CJ extracts, remarkable upregulation of PRX1 and CAT was noted in a dose-dependent manner. The 0.01% and 0.1% CJ groups exhibited significant increase in mRNA expression when compared to that in the H₂O₂ control and the 0.001% CJ

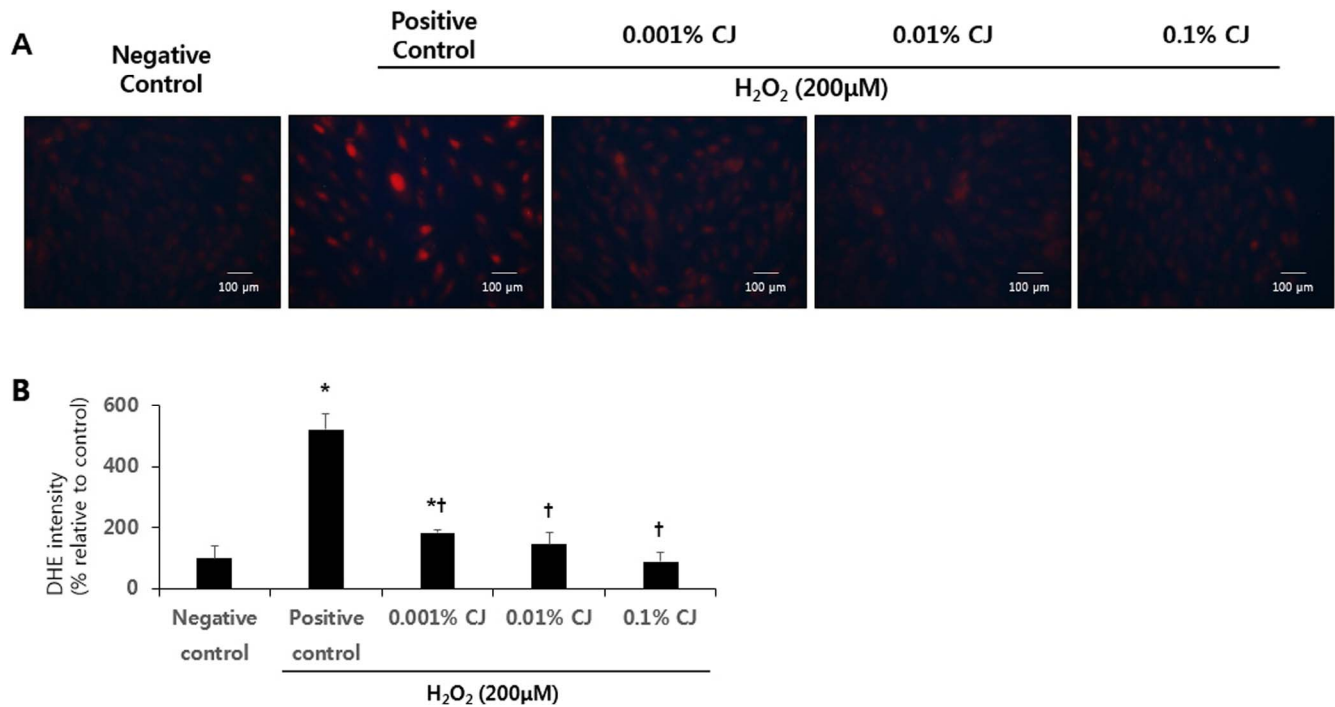


FIGURE 3. Staining with DHE, and the subsequent confocal fluorescence microscopy observations in H₂O₂ (200 µM)-treated HCE cells with or without pretreatment with CJ extracts (0.001%, 0.01%, and 0.1% concentrations). (A) One representative image selected from three individual experiments is shown. (B) Relative fluorescence intensity (expressed as percent normalized to negative control) analysis is shown. **P* < 0.01 compared to the control cells. †*P* < 0.01 compared to the H₂O₂-exposed cells. ‡*P* < 0.05 compared to the 0.001% CJ extract pretreated cells.

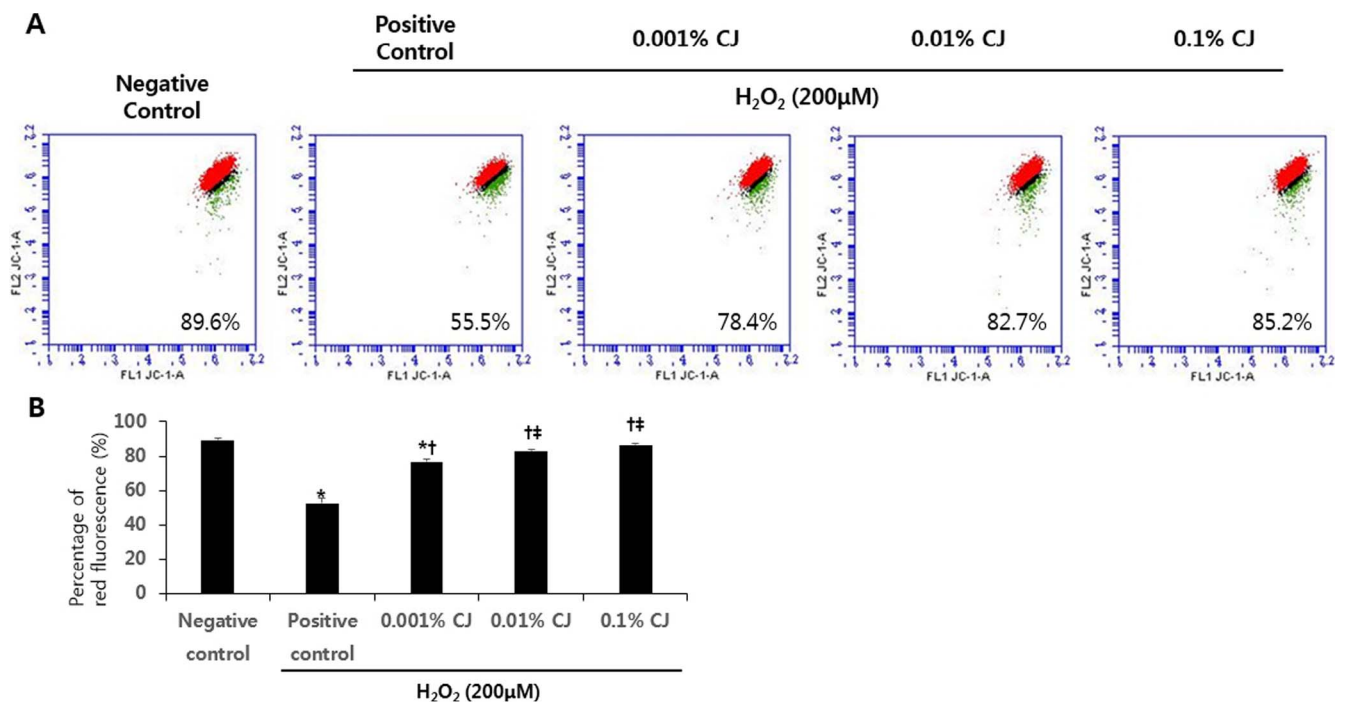


FIGURE 4. Recovery of H₂O₂-induced impairment of mitochondrial membrane potential through treatment with CJ extracts (0.001%, 0.01%, and 0.1% concentration). (A) Representative flow cytometry analysis. One representative data set obtained from among three individual experiments is shown. (B) Percentage of red fluorescence in the naïve control and H₂O₂-treated cells with or without CJ extract pretreatment (0.001%, 0.01%, and 0.1% concentration). **P* < 0.01 compared to the control cells. †*P* < 0.01 compared to the H₂O₂-exposed cells. ‡*P* < 0.05 compared to the 0.001% CJ extract pretreated cells.

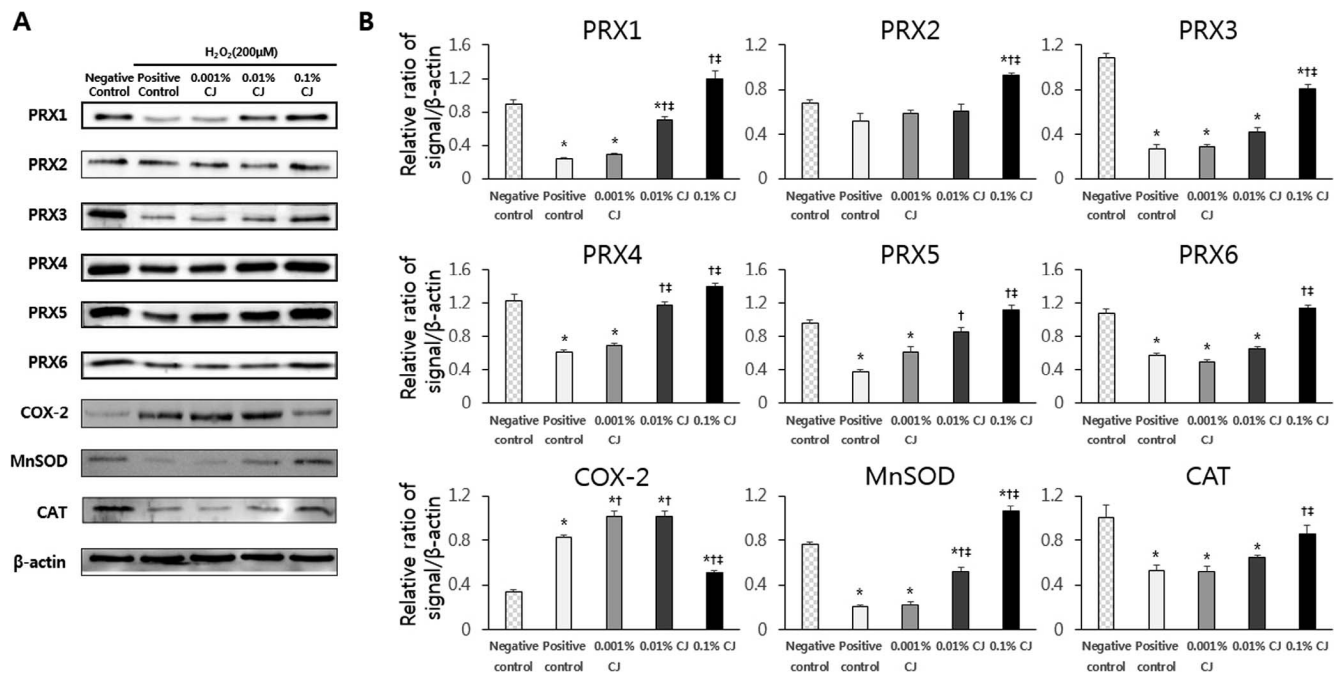


FIGURE 5. Western blot analyses to evaluate the effect of CJ extract on the expression of PRX isoforms, COX-2, MnSOD, and CAT in the presence or absence of H₂O₂ (naïve control, negative control), H₂O₂ control (positive control), and 0.001%, 0.01%, and 0.1% CJ extract groups). β-Actin was used as an internal control. (A) One representative data set obtained from among three individual experiments is shown. (B) Western blot band intensity analyses (relative ratio of signal/β-actin) are shown. **P* < 0.05 compared to the control group, †*P* < 0.05 compared to the H₂O₂ control group, ‡*P* < 0.05 compared to the 0.001% CJ group.

extract group (PRX1, all *P* < 0.01 versus H₂O₂ control and 0.001% CJ; CAT, all *P* = 0.02 versus H₂O₂ control and 0.001% CJ).

Measurement of Tear Volume in the In Vivo EDE Model

Seven days after induction of EDE in the mice, the mean tear volumes were 0.017 ± 0.003 μL and 0.020 ± 0.003 μL in the EDE and BSS groups, respectively (*P* = 0.42). In contrast, the mean tear volumes were 0.024 ± 0.005 μL in the 0.001% CJ extract group (*P* < 0.01 compared to the EDE group; *P* = 0.37 compared to the BSS group), 0.038 ± 0.006 μL in the 0.01% CJ extract group (*P* < 0.01 compared to the EDE and BSS-treated groups), and 0.038 ± 0.006 μL in the 0.1% CJ extract group (*P* < 0.01 compared to the EDE and BSS-treated groups) (Fig. 7).

No differences in the baseline tear volume were observed between the groups (data not shown).

Tear Film BUT

At baseline, the mean tear film BUTs of the five different groups showed no statistically significant differences (data not shown). After 7 days of desiccant stress, the tear film BUT was 1.54 ± 0.22 and 1.59 ± 0.08 seconds in the EDE and BSS groups, respectively (*P* = 0.50; Fig. 8). The 0.1% CJ extract group (1.91 ± 0.08 seconds) showed a significant increase in tear film BUT when compared to that in the EDE-, BSS-, and 0.001% CJ-treated groups (1.62 ± 0.24 seconds; *P* < 0.01, < 0.01, and = 0.03, respectively); however, the 0.01% CJ extract group showed a significant increase in tear film BUT when compared to that in the EDE group only (1.74 ± 0.17 seconds; *P* = 0.04).

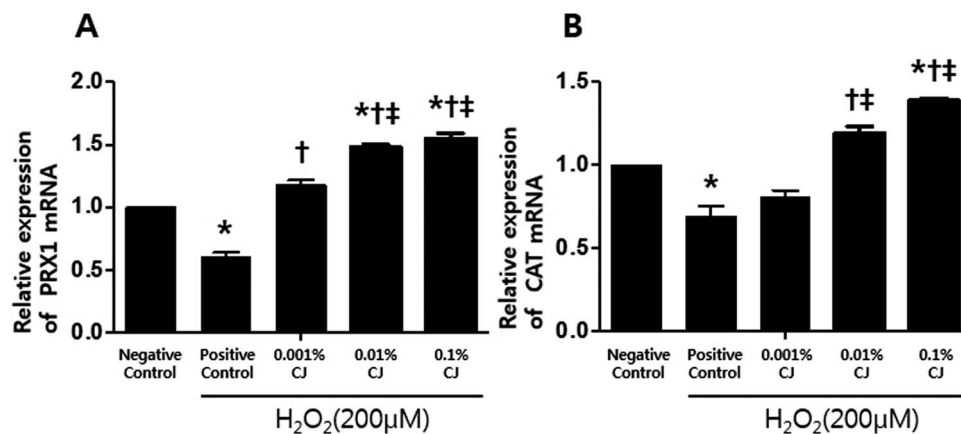


FIGURE 6. The effect of CJ extract (0.001%, 0.01%, and 0.1% concentration) on the mRNA expression levels of (A) PRX1 and (B) CAT in HCE cells after H₂O₂ treatment.

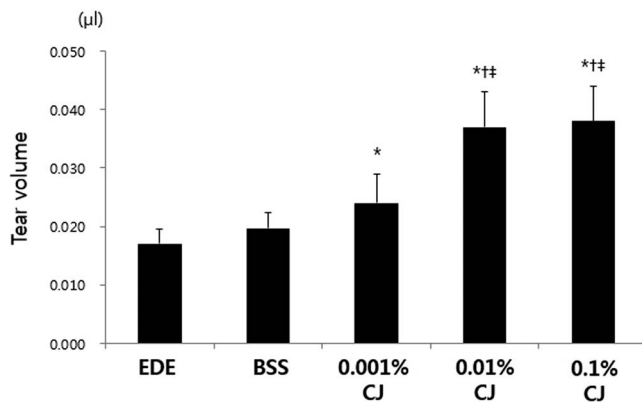


FIGURE 7. Mean tear volumes of the EDE, BSS, and 0.001%, 0.01%, and 0.1% CJ extract-treated groups 7 days after desiccant stress ($n = 15$ per group). * $P < 0.05$ compared to the EDE group. † $P < 0.05$ compared to the BSS group. †† $P < 0.05$ compared to the 0.001% CJ extract-treated group.

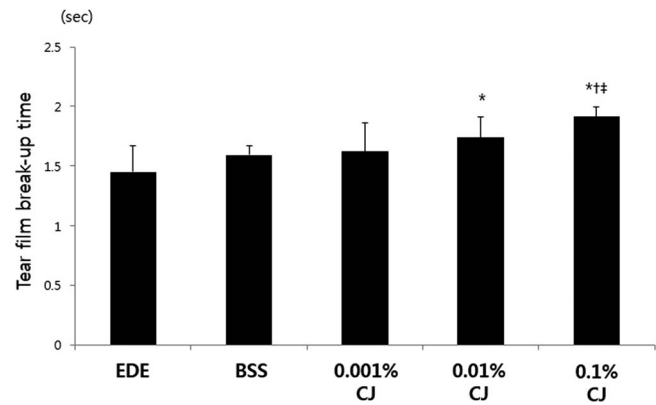


FIGURE 8. Tear film BUT of the EDE, BSS, and 0.001%, 0.01%, and 0.1% CJ extract-treated groups 7 days after desiccant stress ($n = 15$ per group). * $P < 0.05$ compared to the EDE group. † $P < 0.05$ compared to the BSS group. †† $P < 0.05$ compared to the 0.001% CJ extract-treated group.

Corneal Fluorescein Staining

On day 7, the corneal fluorescein staining scores of the EDE and BSS groups were not significantly different (13.20 ± 1.03 vs. 12.50 ± 1.18 , $P = 0.81$). The mean corneal fluorescein staining scores of the three treatment groups were 12.10 ± 0.82 , 11.80 ± 1.03 , and 10.20 ± 0.79 for the 0.001%, 0.01%, and 0.1% CJ extracts, respectively. The 0.1% CJ extract-treated group showed a significant decrease in corneal fluorescein staining score compared to that in the EDE and BSS groups ($P < 0.01$ and $= 0.04$, respectively; Fig. 9). No statistically significant differences in the baseline mean corneal fluorescein staining scores of the five different groups were observed (data not shown).

Inflammatory Cytokine and Chemokine Levels in Conjunctival Tissue

The concentrations of TNF- α , IL-6, IP-10, and MIG in the conjunctivae significantly decreased after instillation of 0.01% and 0.1% CJ extracts for 7 days, in comparison with those of the EDE, BSS, and 0.001% CJ extract groups (all $P < 0.05$; Table 1). The 0.01% CJ extract group showed a significant decrease in IL-1 β concentration when compared to that in the EDE group ($P < 0.01$), and the 0.1% CJ extract group showed a significant decrease in IL-1 β concentration when compared to that in the EDE, BSS, and 0.001% CJ extract groups ($P < 0.01$, < 0.01 , and $= 0.04$, respectively). The levels of IL-6, IP-10, and MIG significantly decreased in the 0.001% CJ extract group when compared to those in the EDE group ($P = 0.03$, < 0.01 , and < 0.01 , respectively). In addition, the IL-6 and IP-10 concentrations of the 0.001% CJ extract group significantly decreased when compared to those in the BSS group ($P = 0.03$ and 0.01 , respectively).

Effect of CJ Extract on Conjunctival Intracellular ROS Level

The fluorescence intensity of DCF-DA was measured. Treatment with 0.001%, 0.01%, and 0.1% CJ extract resulted in a significant decrease in oxidation when compared to that in the EDE group (all $P < 0.01$). Treatment with 0.01% and 0.1% CJ extracts resulted in a significant decrease in oxidation when compared to that in the BSS group (all $P < 0.01$). These results indicated a decrease in the intracellular ROS level in the conjunctival tissue of the CJ treatment groups (Table 2).

DISCUSSION

The pathogenesis of DED is complex with multiple etiologies. Inflammation of the tear-secreting apparatus results in compositional changes in the tear film, and loss of tear film integrity is thought to be one of the important mechanisms leading to ocular surface injury in DED.⁴² Cellular damage in the ocular surface, due to apoptosis and direct mechanical and/or osmotic stress, stimulates the reflex neurosensory arc. This in turn stimulates lacrimal gland and neurogenic inflammation and ultimately inflammation of the ocular surface epithelium.⁴³ These inflammatory processes lead to the release of IL-1, IL-6, IL-8, TNF- α , and other inflammatory cytokines/chemokines from the conjunctival epithelium.¹⁸ Various anti-inflammatory therapies have proven to be useful in the treatment of DED. At present, topical application of cyclosporine has become one of the standard treatments for inflammatory DED.⁴⁴ However, no single treatment successfully manages DED. Hence, the development of new medications with anti-inflammatory properties effective in the management of DED has become an important research target. In accordance with previous studies evaluating the role of inflammation in the pathogenesis of DED, we have proven that materials with anti-inflammatory properties, such as 5-aminoimidazole-4-carboxamide-1- β -d-ribofuranoside, adiponectin, and infliximab, are effective in alleviating the objective parameters of EDE after topical instillation.^{34,45,46} In a continued effort to find substances effective in treating DED, we evaluated the effects of topical instillation of CJ extract in a mouse model of EDE in this study.

Previous studies have proven that CJ extracts demonstrate anti-inflammatory properties by downregulating the expression of pro-inflammatory mediators, such as iNOS, COX-2, and TNF- α .² The anti-inflammatory action of CJ extracts also was shown in this study. Western blot analysis performed during the in vitro study demonstrated that CJ extracts exert anti-inflammatory effects by decreasing the expression of COX-2. The expression of COX-2 is upregulated in the ocular surface after injury and during inflammation.⁴⁷ Quantitative analysis of the Western blot results revealed that the use of 0.1% CJ extract resulted in a significant decrease in the intensity of the COX-2 band when compared to that of the H₂O₂-treated positive control. The anti-inflammatory characteristics of the CJ extracts were more clearly demonstrated during the in vivo study. Topical instillation of CJ extract led to a significant decrease in most of the anti-inflammatory cytokines (e.g., TNF-

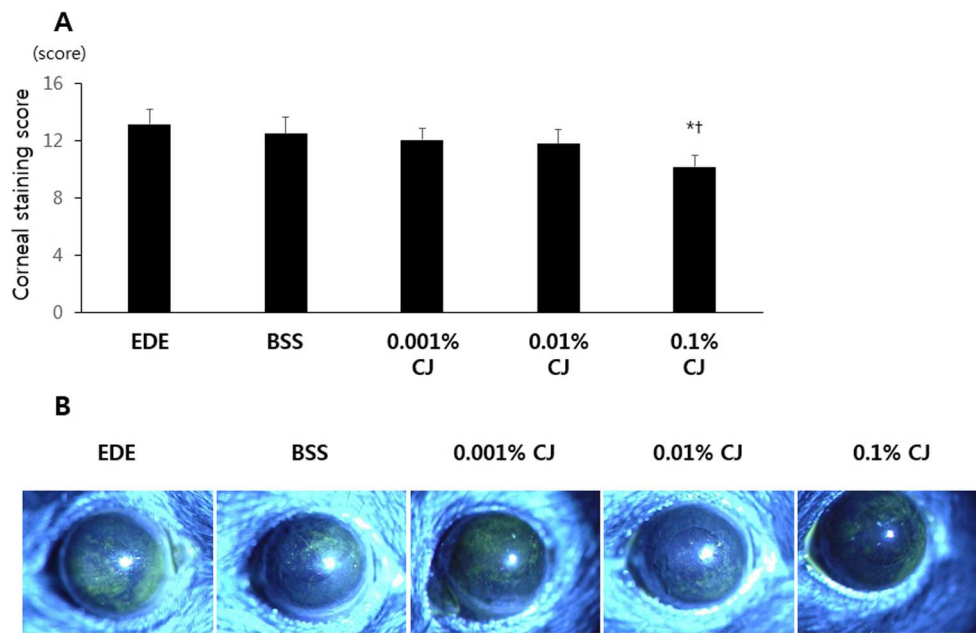


FIGURE 9. Mean corneal fluorescein staining scores (A) and representative figures (B) showing the staining of the EDE, BSS, and 0.001%, 0.01%, and 0.1% CJ extract-treated groups 7 days after desiccant stress ($n = 15$ per group). * $P < 0.05$ compared to the EDE group. † $P < 0.05$ compared to the BSS group.

α , IL-1 β , and IL-6) and chemokines (e.g., IP-10 and MIG) in a dose-dependent manner.

Tumor necrosis factor- α , which activates NF- κ B, is a key transcription factor involved in activating the genes involved in inflammation.¹⁹ Tumor necrosis factor- α also triggers the production of other inflammatory cytokines. Both IL-1 β and IL-6 are proinflammatory cytokines that are secreted on the ocular surface in response to various pathogenic stimuli, such as benzalkonium chloride-induced EDE, desiccant stress-induced EDE, and microbial infections, such as *Pseudomonas aeruginosa*, *Onchocerca volvulus*, and herpes simplex virus 1.^{35,48,49} Interleukin-6, along with other proinflammatory cytokines, has a major role in the pathogenesis of several inflammatory diseases, including DED, graft-versus-host disease, and thyroid-associated ophthalmopathy through the NF- κ B and MAPK pathways.^{50,51} In addition, previous studies have shown that increased expression of IL-1 β during ocular surface inflammation induces the loss of corneal epithelial barrier function.⁵² Inducible protein-10, a member of the α -chemokine subfamily, promotes T-helper type 1 (Th1) immune responses and attracts monocytes and activated T lymphocytes to the inflammatory foci.⁵³ The induction of IP-10 also is NF- κ B-dependent.⁵⁴ Monokine induced by INF- γ is one of the chemokines that attracts Th1 cells. Its expression is increased

on the ocular surface of the murine EDE model as well as in the tear film and on the ocular surface of patients with non-Sjögren's syndrome and Sjögren's syndrome DED.^{20,55} Monokine induced by INF- γ expression is significantly decreased after topical application of anti-inflammatory substances, such as 5-aminoimidazole-4-carboxamide-1- β -d-ribofuranoside and adiponectin.^{34,46} We suspect that inhibition of the NF- κ B signaling pathway by the CJ extracts, as proven by Kim S et al.² led to the decreased expression of anti-inflammatory cytokines and chemokines observed in this work.

Along with its anti-inflammatory properties, CJ is known for its antioxidant and resultant anti-apoptotic activity. This activity may be attributed to its high content of quercetin, quercetin-3-*O*-glucoside, quercitrin, and kaempferol.^{4,12} *Camellia japonica* extracts exert their antioxidant activity by scavenging ROS and enhancing the activity of antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase. During the in vitro study, the antioxidant activity of the CJ extract was proven using the Western blot analysis, which showed increased expression of the PRX family and MnSOD in a dose-dependent manner. The PRX family is a group of antioxidant enzymes that inactivate ROS and thereby protect tissues from oxidative stress. As a major antioxidant enzyme, MnSOD has a crucial role in scavenging O₂⁻.⁵⁶ The

TABLE 1. Concentrations of IFN- γ , IL-1 β , IL-6, TNF- α , IP-10, and MIG in the Conjunctivae of the EDE, BSS, and 0.001%, 0.01%, and 0.1% CJ Extract Groups

Groups	TNF- α	IL-1 β	IL-6	IFN- γ	IP-10	MIG
EDE	18.80 \pm 0.78	40.41 \pm 1.66	22.89 \pm 1.44	3.57 \pm 0.33	21.40 \pm 1.06	46.43 \pm 2.63
BSS	16.23 \pm 0.83	33.15 \pm 3.71	22.12 \pm 3.71	3.38 \pm 0.46	18.6 \pm 0.68	32.79 \pm 2.33*
0.001% CJ	16.41 \pm 0.61	29.41 \pm 1.95	16.86 \pm 1.20*†	3.23 \pm 0.22	14.45 \pm 0.76*†	33.32 \pm 1.22*
0.01% CJ	7.91 \pm 0.87*†‡	22.97 \pm 1.25*	10.37 \pm 1.25*†‡	3.17 \pm 0.19	6.00 \pm 1.01*†‡	11.78 \pm 0.97*†‡
0.1% CJ	3.53 \pm 0.50*†‡	16.89 \pm 1.53*†‡	9.47 \pm 0.48*†‡	3.05 \pm 0.17	3.82 \pm 0.76*†‡	9.41 \pm 0.76*†‡

Data are expressed as mean \pm SD.

* $P < 0.05$ compared to the EDE group.

† $P < 0.05$ compared to the BSS group.

‡ $P < 0.05$ compared to the 0.001% CJ group.

TABLE 2. Conjunctival ROS Levels in the EDE, BSS, and 0.001%, 0.01%, and 0.1% CJ Extract Groups

Groups	ROS, DCF Fluorescein Intensity
EDE	191.8 ± 12.1
BSS	165.8 ± 10.3
0.001% CJ	125.0 ± 3.6*
0.01% CJ	120.3 ± 5.0*†
0.1% CJ	112.5 ± 7.9*†

Data are expressed as mean ± SD.

* $P < 0.05$ compared to the EDE group.

† $P < 0.05$ compared to the BSS group.

expression of CAT, which is a well-known protector from oxidative stress, also increased after pretreatment with CJ extract. Furthermore, the DCF-DA and DHA analyses and the mitochondrial membrane potential change measurements showed that application of CJ extract exerts an antiapoptotic effect in a dose-dependent manner. These findings demonstrated that CJ extract could protect HCE cells from oxidative damage by reducing ROS levels while increasing the production of antioxidant enzymes, thus restoring the balance between oxygenases and antioxidant enzymes. The antioxidant properties of CJ extracts also were demonstrated during the in vivo study using a murine EDE model. The level of conjunctival intracellular ROS was significantly decreased following topical instillation of CJ extract. Accumulating evidence suggests that oxidative stress has an important role in ocular surface diseases, including DED.^{40,57,58} In vivo lipid oxidative injury has been observed in DED and Sjögren's syndrome patients.^{59,60} In vitro studies further revealed that lipid and mitochondrial oxidative damage causes inflammation in HCE cells.^{39,61}

As mentioned above, various anti-inflammatory drugs, such as topical steroids and cyclosporine, have been widely used to treat DED, and studies have shown that anti-inflammatory treatment has a beneficial effect on tear secretion, tear film stability, and ocular surface integrity.^{62,63} In addition, as oxidative stress has a potent role in the pathogenesis of DED, antioxidative treatment has shown therapeutic effects against DED, proven by the improvement in various ocular surface parameters.⁶¹ Therefore, we further investigated the effects of CJ extract on various clinical parameters, including tear volume, tear film BUT, and corneal fluorescein staining in a mouse model of EDE. Despite continuous exposure to desiccant stress and rigorous anticholinergic treatment, 0.01% and 0.1% CJ extract-treated eyes showed an improved tear film BUT and increased tear production. In addition, the 0.01% CJ extract group showed reversal of corneal epithelial damage, which was indicated by the decreased fluorescein staining when compared to that of the untreated eyes and eyes treated with just the vehicle. Our study demonstrated that the protective effects of CJ extract against oxidative stress, apoptosis, and inflammation of the ocular surface had beneficial effect on the improvement of various tear film and ocular surface parameters.

The limitation of our study is that the experiment was conducted only with supercritical extracts of CJ leaves without extensive investigation of the component's phytochemical substances. Additional studies with fractionated extracts are needed to elucidate the active substances in the CJ extracts. Another limitation is that analysis of the ocular surface microstructure (e.g., corneal nerves or cell morphology) was not conducted during the in vivo part of our study. Further studies on the effect of CJ extract on the structural and functional changes of the ocular surface are needed.

To the best of our knowledge, this study is the first to investigate the possibility of using CJ extracts for topical application. Taking our findings into consideration, as well as those of the aforementioned studies, we suggest that topical CJ extract application, especially at concentrations of 0.01% and 0.1%, ameliorates DED, as determined by clinical, inflammatory, and oxidative measurements. The results presented support the idea that CJ extracts have potential as an adjunctive therapeutic agents for the treatment of dry eye.

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