Effect of Topical 5-Aminoimidazole-4-carboxamide-1-β-d-Ribofuranoside in a Mouse Model of Experimental Dry Eye

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Purpose. To investigate the efficacy of topical 5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside (AICAR) in a mouse model of experimental dry eye (EDE).

Methods. Eye drops consisting of 0.001% or 0.01% AICAR, 0.05% cyclosporine A (CsA), or balanced salt solution (BSS) were applied for the treatment of EDE. Tear volume, tear film break-up time (BUT), and corneal fluorescein staining scores were measured 10 days after treatment. Levels of interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interferon gamma-induced protein 10 (IP-10), and monokine induced by interferon-γ (MIG) were measured in the conjunctiva. In addition, Western blot, periodic acid-Schiff staining for evaluating goblet cell density, flow cytometry for counting the number of CD4+CXCR3+ T cells, and immunohistochemistry for detection of 4-hydroxy-2-nonenal (4HNE) were performed.

Results. Mice treated with 0.01% AICAR showed a significant improvement in all clinical parameters compared with the EDE control, vehicle control, and 0.001% AICAR groups ($P < 0.001$). A significant decrease in the levels of IL-1β, IL-6, TNF-α, IFN-γ, IP-10, and MIG, the number of CD4+CXCR3+ T cells, and the number of 4HNE-positive cells were also observed in the 0.01% AICAR group ($P < 0.001$). Although 0.05% CsA also led to an improvement in clinical parameters and inflammatory molecule levels, its therapeutic effects were comparable or inferior to those of 0.01% AICAR.

Conclusions. Topical application of 0.01% AICAR can markedly improve clinical signs and decrease inflammation in the ocular surface of EDE, suggesting that AICAR eye drops may be used as a therapeutic agent for dry eye disease.

Keywords: 5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside (AICAR), experimental dry eye, AMP-activated protein kinase (AMPK), anti-inflammation

Dry eye disease (DED) is a chronic ocular disorder affecting 10% to 20% of the world's population.[1–3] The pathology of DED is closely related to inflammation in the cornea and the conjunctiva, in which T cells are highly involved.[4–6] According to the definition and classification of dry eye proposed by the Dry Eye Workshop, DED is a multifactorial disorder in which inflammation plays a relevant role.[7] At present, cyclosporine A (CsA) and steroids, which have anti-inflammatory effects, are frequently used to treat DED. In addition, new treatment approaches are also being designed to address the underlying disease process: inflammation on the ocular surface.

Adenosine monophosphate (AMP)–activated protein kinase (AMPK) is a serine/threonine protein kinase that has emerged as a master sensor of cellular energy balance in mammalian cells.[8,9] It is activated when cells experience energy-depleting stresses.[10] This protein kinase exists as a heterotrimeric enzyme consisting of a catalytic subunit (α) and two regulatory subunits (β and γ), and its activity is dependent on the phosphorylation at a major activating site (Thr172) of the α-subunit. In addition to its role in metabolic processes, AMPK was also implicated as an anti-inflammatory target. Several cellular and animal models have demonstrated that anti-inflammatory effects are mediated by AMPK. The benefits of AMPK activation in several inflammatory disease models have also been documented.[11–16]

Widely used as a pharmacologic activator of AMPK, 5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside (AICAR) is absorbed into cells and converted to the monophosphorylated form 5-aminoimidazole-4-carboxamide ribonucleoside (ZMP) by adenosine kinase, mimicking an increase in intracellular AMP levels.[17] Recently, the anti-inflammatory effects of AICAR have been reported in disease states including ischemia and reperfusion heart injury, acute lung injury, and some autoimmune diseases.[15,18,19] In ophthalmological practice, intraperitoneal AICAR injections have been shown to suppress uveitis-related intraocular inflammation and increase tear secretion volume in mice.[20–22]

In the present study, we hypothesized that topical application of AICAR would affect ocular surface inflammation in dry eye. Hence, we investigated the effects of AICAR eye drops on the various clinical parameters and inflammatory molecules on the ocular surface in a mouse model of experimental dry eye (EDE).

Methods

Mouse Model of Dry Eye and Experimental Procedures

This research protocol was approved by the Chonnam National University Medical School Research Institutional Animal Care and Use Committee. All animals were treated in accordance with the ARVO 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., St. Louis, MO, USA) four times a day (8 AM, 11 AM, 2 PM, and 5 PM) with exposure to an air draft and 30% ambient humidity, as previously described.[23–25] During these experiments, animal behavior, food, and water intake were not restricted.

The mice were randomly divided into six groups according to the topical treatment administered as follows: (1) untreated control (UT): mice that were not exposed to desiccating stress or treated topically; (2) EDE control: mice that received no eye drops; (3) vehicle control: EDE mice treated with balanced salt solution (BSS; Alcon, Fort Worth, TX, USA); (4) EDE mice treated with 0.05% CsA (Restasis; Allergan, Irvine, CA, USA); (5) EDE mice treated with 0.001% AICAR; and (6) EDE mice treated with 0.01% AICAR. AICAR (Toronto Research Chemicals, Toronto, ON, Canada) was diluted in BSS. Two microliters of the eye drops were applied topically to both eyes of unanesthetized mice three times a day (8 AM, 12 PM, 5 PM), daily until they were killed. Clinical parameters, including tear volume, tear film break-up time (BUT), and corneal fluorescein staining scores, were measured 10 days after treatment. The clinical measurements were made after 3 hours of the last scopolamine injection and eye drops application. After measurement of the clinical parameters, the mice were euthanized, and Western blot, multiplex immunobead assay, histology, flow cytometry, and immunohistochemistry were performed. Each group consisted of five animals, and the experiments were performed on three independent sets of mice.

Tear Volume Measurements

Tear volume was measured using phenol red-impregnated cotton threads (Zone-Quick; Oasis, Glendora, CA, USA) after 3 hours of the last scopolamine injection, as previously described.[26] The threads were placed in the lateral canthus for 20 seconds. Tear volume, expressed in millimeters of thread wet by the tear and turned red, was measured using a microscope (SMZ 1500; Nikon, Melville, NY, USA). A standard curve was derived to convert distance into volume.

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

We dropped 1 μL of 1% sodium fluorescein into the inferior conjunctival sac using a micropipette. After three blinks, tear film BUT was recorded in seconds using slit lamp biomicroscopy (BQ-900; Haag-Streit, Bern, Switzerland) under cobalt blue light. Ninety seconds later, punctate staining on the corneal surface was evaluated in a masked fashion. Each cornea was divided into four quadrants that were scored individually. Corneal fluorescein staining severity score was calculated using a 4-point scale: 0, absent; 1, slightly punctate staining $<$30 spots; 2, punctate staining $>$30 spots, but not diffuse; 3, severe diffuse staining but no positive plaque; and 4, positive fluorescein plaque.[27] The four scores were added to generate a final grade (possible total of 16 points).

Western Blot

Proteins were extracted from the conjunctival tissues by using a lysis buffer (M-PER; Pierce Biotechnology, Rockford, IL, USA) with protease inhibitor cocktail. The lysates were centrifuged at 15,000 rpm for 10 minutes at 4°C. The proteins (40 μg) of the samples were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. The blots were then washed with TBST (10 mM Tris-HCl [pH 7.6], 150 mM NaCl, 0.05% Tween-20), blocked with 5% skim milk in TBST for 1 hour and incubated for 2 hours at room temperature with primary antibodies, which included rabbit anti-AMPKα, rabbit anti-phospho-AMPKα, rabbit anti-NF-κB p65, or rabbit anti-phospho-NF-κB p65 (primary antibodies obtained from Cell Signaling Technology, Beverly, MA, USA). After incubating with secondary antibodies, the immunoreactive bands were visualized using an enhanced chemiluminescence system (ECL Blotting Analysis System; Amersham, Arlington Heights, IL, USA). Rabbit anti-β-actin was used as an inner control.

Multiplex Immunobead Assay

A multiplex immunobead assay (Luminex 200; Luminex Corp., Austin, TX, USA) was used to measure concentrations of interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, IFN- γ-induced protein (IP)-10, and monokine induced by IFN- γ (MIG) in the conjunctiva, as previously described.[28] The tissues were collected and pooled in lysis buffer containing protease inhibitors for 30 minutes. The cell extracts were centrifuged at 14,000*g* for 15 minutes at 4°C, and the supernatants were stored at −70°C until use. Total protein concentration in supernatants was determined, and 25 μL of total protein of each sample was pipetted into assay plate wells. The supernatants were added to wells containing the appropriate cytokine bead mixture that included mouse monoclonal antibodies specific for the cytokines and chemokines for 60 minutes. After three washes, the biotinylated secondary antibody mixture was applied for 30 minutes in the dark at room temperature. The reactions were detected after addition of streptavidin-phycoerythrin with an analysis system (xPONENT; Luminex Corp.). The concentrations of the cytokines and chemokines in tissues were calculated from standard curves of known concentrations of recombinant mouse cytokines.

Histology

Conjunctival tissue was surgically excised, fixed in 4% paraformaldehyde, and embedded in paraffin. We stained 6-μm sections with periodic acid-Schiff (PAS) reagent. Sections from four animals from each group were examined and photographed with a microscope (Olympus Corp., Tokyo, Japan) equipped with a digital camera. Goblet cell density in the superior and inferior conjunctiva was measured in three sections from each eye using image analysis software (Media Cybernetics, Silver Spring, MD, USA) and was expressed as the number of goblet cells per 100 μm.

Flow Cytometry

Flow cytometry was performed to count the number of CD4+ CXCR3+ T cells from the conjunctiva using a previously

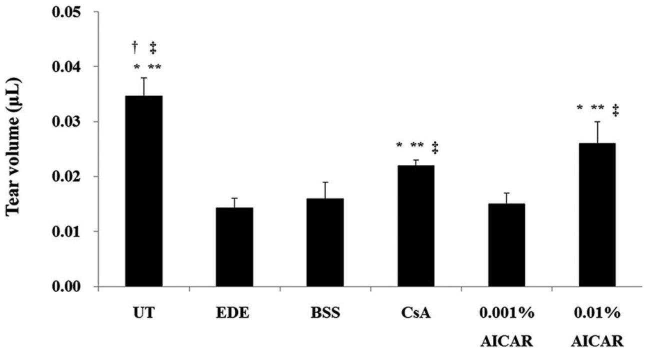

FIGURE 1. Mean tear volumes in the UT, EDE control, BSS-treated, 0.05% CsA-treated, 0.001% AICAR-treated, and 0.01% AICAR-treated groups at day 10. *$P < 0.05$ compared with the EDE group. **$P < 0.05$ compared with the BSS group. †$P < 0.05$ compared with the CsA group. ‡$P < 0.05$ compared with the 0.001% AICAR group.

described method.[29] Tissues from each group were harvested, dipped in PBS, teased apart with scissors, and shaken at 37°C for 60 minutes in the presence of 0.5 mg/mL collagenase type D (Roche Applied Science, Indianapolis, IN, USA). After incubation, the tissues were disrupted by grinding with a syringe plunger and passed through a cell strainer with a pore size of 100 μm. Cells were centrifuged at 1500 revolutions per minute for 7 minutes and resuspended in PBS with 1% bovine serum albumin. After washing, the samples were incubated with fluorescein-conjugated anti-CD4 antibody (BD Biosciences, San Jose, CA, USA); phycoerythrin-conjugated anti-CXCR3 antibody (clone 173; BD Biosciences); and isotype control antibody at 37°C for 30 minutes. Phycoerythrin-conjugated rat IgG isotype (BD Biosciences) was used as the control. The number of CD4+ CXCR3+ T cells was counted using the FACSCalibur cytometer with CellQuest software (BD Bioscience).

Immunohistochemistry

Oxidative stress-induced lipid peroxidation was assessed by immunohistochemical detection of 4-hydroxy-2-nonenal (4HNE) in the conjunctiva. Tissues were fixed overnight in a 4% buffered paraformaldehyde solution and processed for paraffin embedding. We cut 6-μm sections from paraffin wax blocks, mounted on precoated glass slides, deparaffinized, and rehydrated. Hydrogen peroxide (H_2O_2; 3%) in PBS and 1% serum in PBS were sequentially applied to the sections. Conjunctival sections were incubated with mouse anti-4HNE monoclonal antibody (JaICA, Shizuoka, Japan) at a concentration of 25 μg/mL for 1 hour at room temperature. After washing, the secondary antibodies were applied. The samples were incubated with avidin-peroxidase, then incubated with 3,3′-diaminobenzidine peroxidase substrate and counterstained with Mayer's hematoxylin. The number of cells positively stained for 4HNE per 100 μm was calculated.

Statistical Analysis

Commercial software (SPSS version 18.0; SPSS, Inc., Chicago, IL, USA) was used for statistical analyses. Statistical differences in tear volume, tear film BUT, and corneal fluorescein staining among the groups was determined by one-way ANOVA test with Turkey post hoc analysis. The Kruskal-Wallis and Mann-Whitney *U* test were used to compare cytokine and chemokine levels, goblet cell density, 4HNE-positive cell density, and flow cytometry differences between the groups. A value of $P < 0.05$ was considered statistically significant.

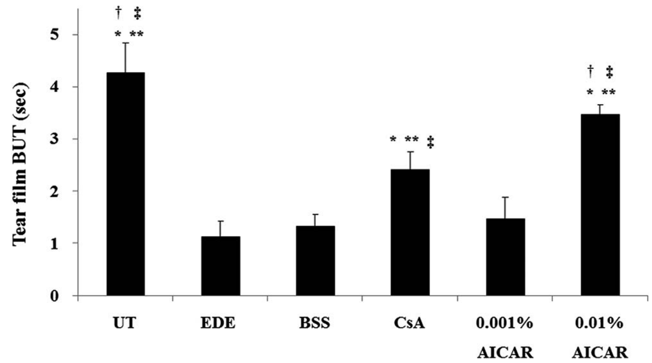

FIGURE 2. Tear film BUT in the UT, EDE control, BSS-treated, 0.05% CsA-treated, 0.001% AICAR-treated, and 0.01% AICAR-treated groups at day 10. *$P < 0.05$ compared with the EDE group. **$P < 0.05$ compared with the BSS group. †$P < 0.05$ compared with the CsA group. ‡$P < 0.05$ compared with the 0.001% AICAR group.

RESULTS

Tear Volume

There were no statistically significant differences in tear volumes among the groups at baseline (data not shown). Ten days after desiccating stress, the mean tear volume significantly decreased in the EDE group (0.014 ± 0.002 μL) compared with the UT group (0.035 ± 0.003 μL; $P < 0.001$). The mean tear volumes of four treatment groups were 0.016 ± 0.003 μL, 0.022 ± 0.001 μL, 0.015 ± 0.002 μL, and 0.026 ± 0.004 μL in the BSS, CsA, 0.001% AICAR, and 0.01% AICAR groups, respectively. The cyclosporine A and 0.01% AICAR groups showed significant increases in tear volume compared with the EDE, BSS and 0.001% AICAR group (all $P < 0.001$). There was no difference in tear volume between the CsA and 0.01% AICAR groups ($P = 0.061$; Fig. 1).

Tear Film BUT

There were no statistically significant differences in tear film BUT among the groups at baseline (data not shown). Ten days after desiccating stress, tear film BUT was significantly shorter in the EDE group (1.13 ± 0.31 seconds) compared with the UT group (4.27 ± 0.57 seconds; $P < 0.001$). The cyclosporine A (2.42 ± 0.34 seconds) and 0.01% AICAR (3.47 ± 0.19 seconds) groups showed a significant increase in tear film BUT compared with the EDE, BSS and the 0.001% AICAR groups (all $P < 0.001$), while the BSS and 0.001% AICAR group (1.33 ± 0.23 and 1.47 ± 0.42 seconds, respectively) showed no significant improvement compared with the EDE group ($P = 0.461$ and 0.251, respectively). Moreover, statistically significant improvements were observed in tear film BUT in the 0.01% AICAR group compared with the CsA group ($P < 0.001$; Fig. 2).

Corneal Fluorescein Staining

At baseline, the mean corneal fluorescein staining scores in the six different groups showed no statistically significant differences. On day 10, the corneal fluorescein staining scores in the UT, EDE, and BSS groups were 2.08 ± 0.86, 14.10 ± 1.10, and 13.60 ± 1.55, respectively. Treatment with topical CsA, 0.001% AICAR, and 0.01% AICAR led to a significant decrease in corneal staining compared with the untreated EDE group (8.62 ± 2.12, 12.00 ± 1.41, and 6.60 ± 1.78, respectively; $P < 0.001$, $P = 0.039$, and $P < 0.001$, respectively). Moreover, the CsA and 0.01% AICAR groups showed a greater reduction in corneal staining score compared with the vehicle-controlled BSS and 0.001% AICAR

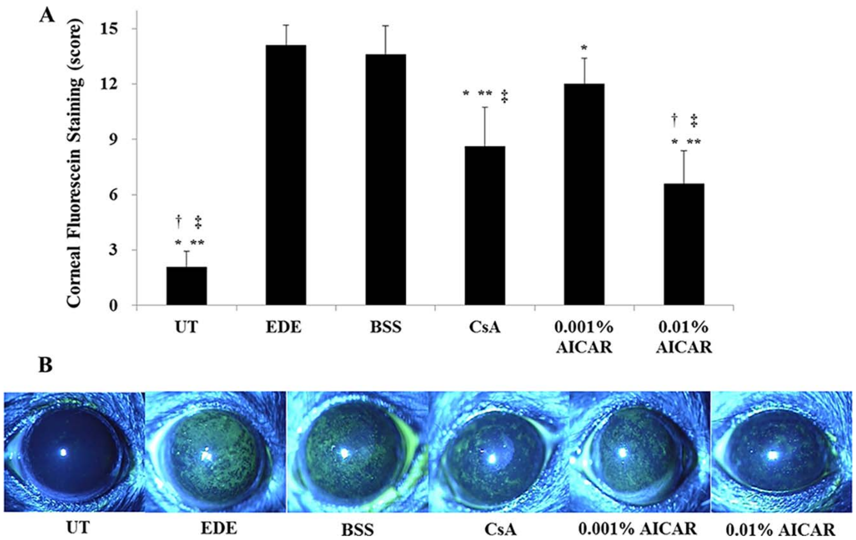

FIGURE 3. Corneal fluorescein staining scores (**A**) and representative figures (**B**) in the UT, EDE control, BSS-treated, 0.05% CsA-treated, 0.001% AICAR-treated, and 0.01% AICAR-treated groups at day 10. All treated groups demonstrate significantly less corneal damage compared to the EDE group. The 0.01% AICAR treatment led to a significant decrease in corneal fluorescein staining compared with the EDE, CsA, and 0.001% AICAR groups. **P* < 0.05 compared with the EDE group. ***P* < 0.05 compared with the BSS group. †*P* < 0.05 compared with the CsA group. ‡*P* < 0.05 compared with the 0.001% AICAR group.

group (all $P < 0.001$). Compared with the CsA and 0.01% AICAR groups, the 0.01% AICAR group showed a greater improvement in corneal staining ($P = 0.042$; Figs. 3A, 3B).

Activation of AMPK and NF-κB in Conjunctival Tissue

To investigate involvement of AMPK and NF-κB activation, we identified the expression of phosphorylated AMPKα (p-AMPKα) and phosphorylated NF-κB p65 (p-NF-κB p65) in conjunctival tissue (Figs. 4A, 4B). In the EDE group, we found that the ratio of activated (p-AMPKα) to total AMPKα (t-AMPKα) was reduced and the ratio of activated (p-NF-κB p65) to total NF-κB p65 (t-NF-κB p65) was increased. Treatment with 0.001% and 0.01% AICAR reversed the conjunctival AMPK deactivation and suppressed the NF-κB activation. These effects were more pronounced in the 0.01% AICAR group than in the 0.001% AICAR group.

Inflammatory Cytokine and Chemokine Levels in Conjunctival Tissue

The results of inflammatory cytokine and chemokine levels in conjunctival tissues are shown in Figure 5. The concentrations

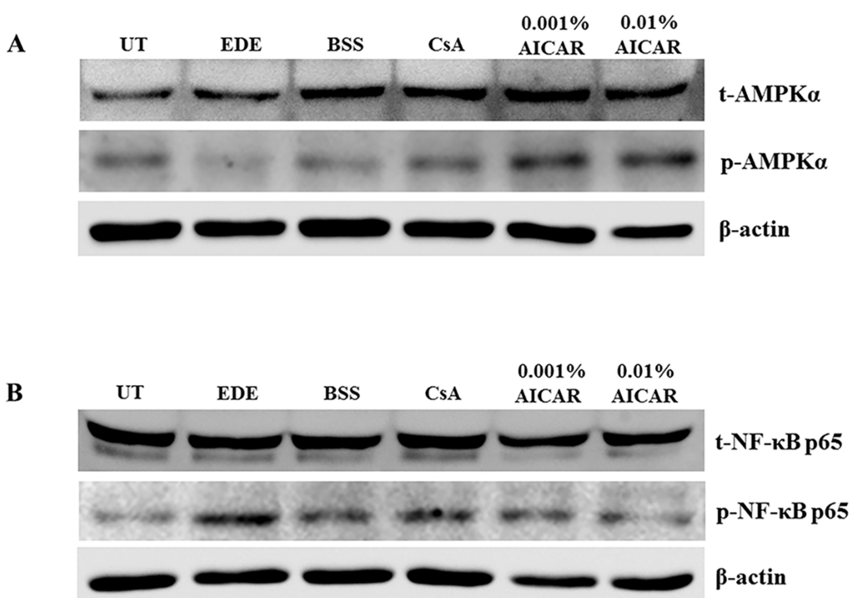

FIGURE 4. Western blot analysis of AMPK and NF-κB activation in the UT, EDE control, BSS-treated, 0.05% CsA-treated, 0.001% AICAR-treated, and 0.01% AICAR-treated groups at day 10. (**A**) Representative immunoblots of cell lysates showing detection of phosphorylated AMPKα (p-AMPKα) and total AMPKα (t-AMPKα) with β-actin loading control. (**B**) Representative immunoblots of cell lysates showing detection of phophorlyated NF-κB p65 (p-NF-κB p65) and total NF-κB p65 (t-NF-κB p65) with β-actin loading control.

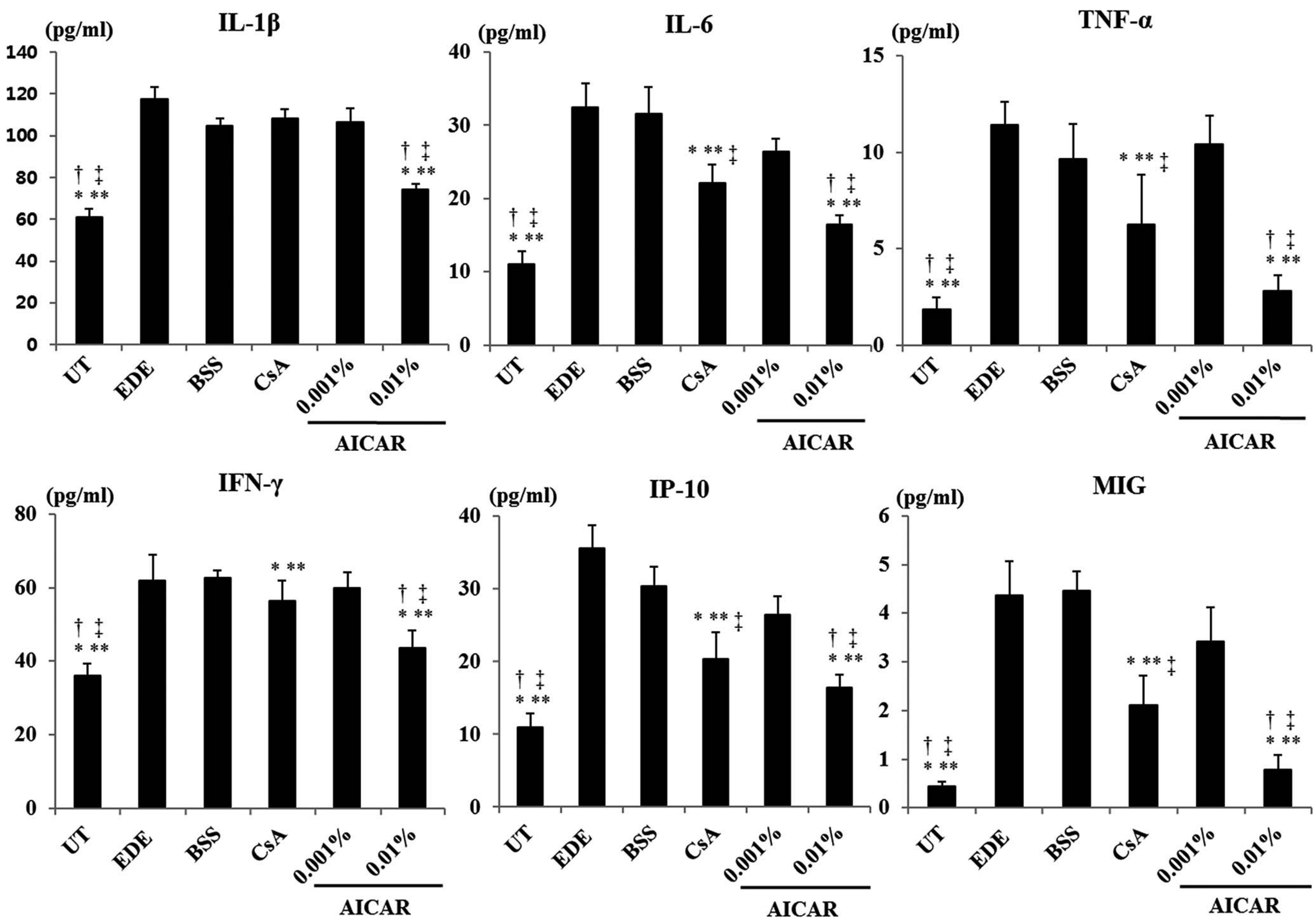

FIGURE 5. Concentrations of IL-1β, IL-6, TNF-α, IFN-γ, IFN- γ-IP-10, and MIG in the UT, EDE control, BSS-treated, 0.05% CsA-treated, 0.001% AICAR-treated, and 0.01% AICAR-treated groups at day 10. *$P < 0.05$ compared with the EDE group. **$P < 0.05$ compared with the BSS group. †$P < 0.05$ compared with the CsA group. ‡$P < 0.05$ compared with the 0.001% AICAR group.

of IL-1β, IL-6, TNF-α, IFN-γ, IP-10, and MIG in the conjunctiva increased 10 days after induction of EDE (all $P < 0.001$), and 0.01% AICAR significantly suppressed these elevations (all $P < 0.001$). The group with CsA also significantly suppressed IL-6, TNF-α, IFN-γ, IP-10, and MIG levels, compared with the EDE control group ($P < 0.001$, $P < 0.001$, $P = 0.026$, $P < 0.001$, and $P < 0.001$, respectively). No differences in inflammatory cytokine and chemokine levels were observed between the 0.001% AICAR and EDE group. Additionally, the 0.01% AICAR group showed a greater reduction of these inflammatory molecules compared with the CsA group (all $P < 0.001$).

Conjunctival Goblet Cell Density

The mean density of conjunctival goblet cells significantly decreased after 10 days of desiccating stress in the EDE group (10.80 ± 2.39 cells/100 μm) compared with the UT group (21.60 ± 2.55 cells/100 μm; $P < 0.001$). The mean goblet cell densities were 10.52 ± 3.57 cells/100 μm, 15.90 ± 3.32 cells/100 μm, 11.60 ± 2.41 cells/100 μm, and 19.40 ± 2.68 cells/100 μm in the BSS, CsA, 0.001% AICAR, and 0.01% AICAR groups, respectively. The cyclosporine A and 0.01% AICAR groups showed significantly higher goblet cell densities than the EDE ($P = 0.002$ and $P < 0.001$, respectively), BSS (both $P < 0.001$), and 0.001% AICAR groups ($P = 0.007$ and $P < 0.001$, respectively). In addition, there was a significant difference in goblet cell density between the CsA and 0.01% AICAR groups ($P = 0.018$; Figs. 6A, 6B).

Flow Cytometric Analysis

Histograms of percentages of CD4+ CXCR3+ T cells from representative samples from the UT, EDE, BSS, CsA, 0.001% AICAR, and 0.01% AICAR groups are shown in Figure 7. The respective percentages of CD4+CXCR3+ T cells were 16.05% ± 5.22%, 64.36% ± 15.19%, 59.37% ± 14.64%, 36.48% ± 7.65%, 58.18% ± 16.26%, and 31.47% ± 10.47%, respectively. The number of Th1 CD4+ T cells significantly decreased in the CsA and 0.01% AICAR groups compared with the EDE, BSS and 0.001% AICAR groups (all $P < 0.001$). There was no significant difference in the percentages of CD4+ CXCR3+ T cells between the CsA and 0.01% AICAR groups ($P = 0.251$).

Quantification of the Oxidative Stress Marker 4HNE in Conjunctival Tissue

Since AICAR has also been shown to have antioxidative properties,[30,31] we made further efforts to investigate oxidative damage through 4HNE immunohistochemical staining. The number of cells positively stained for 4HNE were 9.35 ± 3.62 cells/100 μm, 28.21 ± 5.16 cells/100 μm, 22.35 ± 2.56 cells/100 μm, 22.27 ± 4.55 cells/100 μm, 23.48 ± 6.27 cells/100 μm, and 13.06 ± 3.98 cells/100 μm in the UT, EDE, BSS, CsA, 0.001% AICAR, and 0.01% AICAR groups, respectively. Although 0.05% CsA and 0.001% AICAR treatment did not lead to an improvement in oxidative damage status, the 0.01% AICAR group showed a significant decrease in the number of

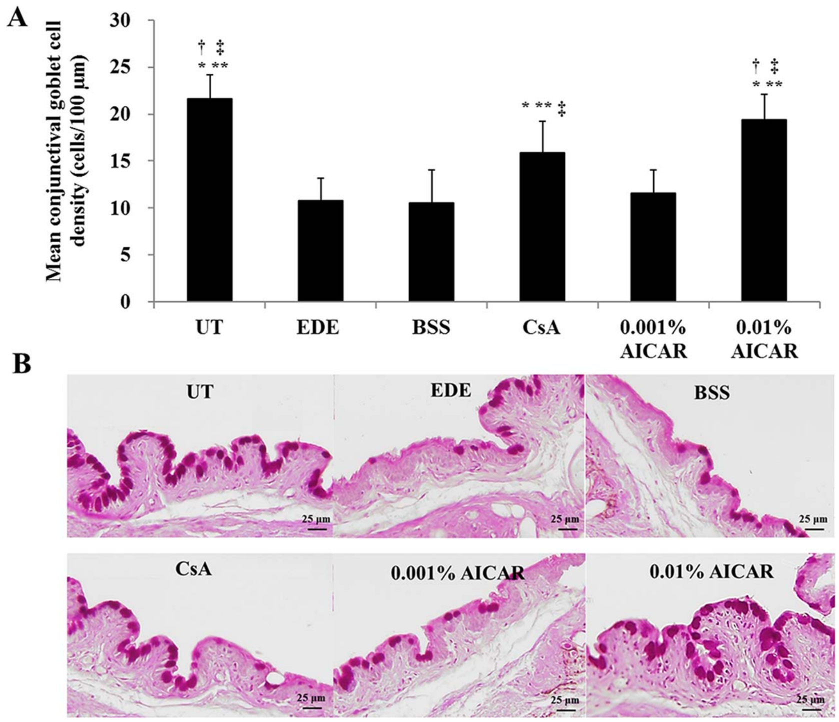

FIGURE 6. Mean goblet cell densities (A) and PAS staining of representative specimens (B) in the UT, EDE control, BSS-treated, 0.05% CsA-treated, 0.001% AICAR-treated, and 0.01% AICAR-treated groups after 10 days of treatment. The 0.01% AICAR-treated group shows a significantly higher number of goblet cells compared with the EDE, CsA, and 0.001% AICAR-treated groups. *$P < 0.05$ compared with the EDE group. **$P < 0.05$ compared with the BSS group. †$P < 0.05$ compared with the CsA group. ‡$P < 0.05$ compared with the 0.001% AICAR group. *Scale bars*: 25 µm.

cells positively stained for 4HNE on the ocular surface ($P < 0.001$; Figs. 8A, 8B).

DISCUSSION

It is generally agreed that DED has a definite correlation with local inflammatory processes in the lacrimal functional unit composed of the ocular surface epithelium and lacrimal glands. Hence, the underlying inflammatory processes should be assessed in detail for effective treatment of DED. Recently, among various treatment options, 0.05% CsA has become one of the standard treatments for inflammatory DED.[32] Cyclosporine A has been shown to inhibit epithelial apoptosis and cytokine production by activated T lymphocytes that infiltrate the conjunctiva, leading to a decrease in inflammation and an increase in tear production, both effects being highly beneficial for the treatment of DED.[33,34] However, CsA use is associated with tolerability issues, such as stinging and burning in some patients.[32,33,35] Recently, development of a new drug with anti-inflammatory action for the treatment of dry eye has become an important target of research.

We previously reported the therapeutic effect of adiponectin eye drops in a mouse model of EDE through activation of AMPK and inhibition of various proinflammatory signaling

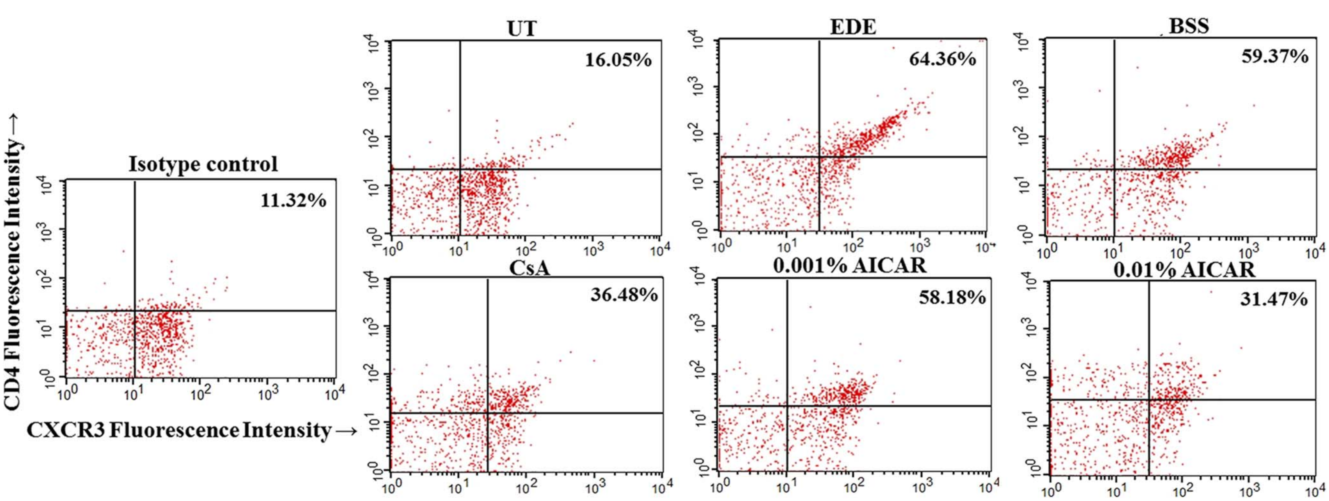

FIGURE 7. Flow cytometry showing CD4+ CXCR3+ T cells in the conjunctiva of the UT, EDE control, BSS-treated, 0.05% CsA-treated, 0.001% AICAR-treated, and 0.01% AICAR-treated groups.

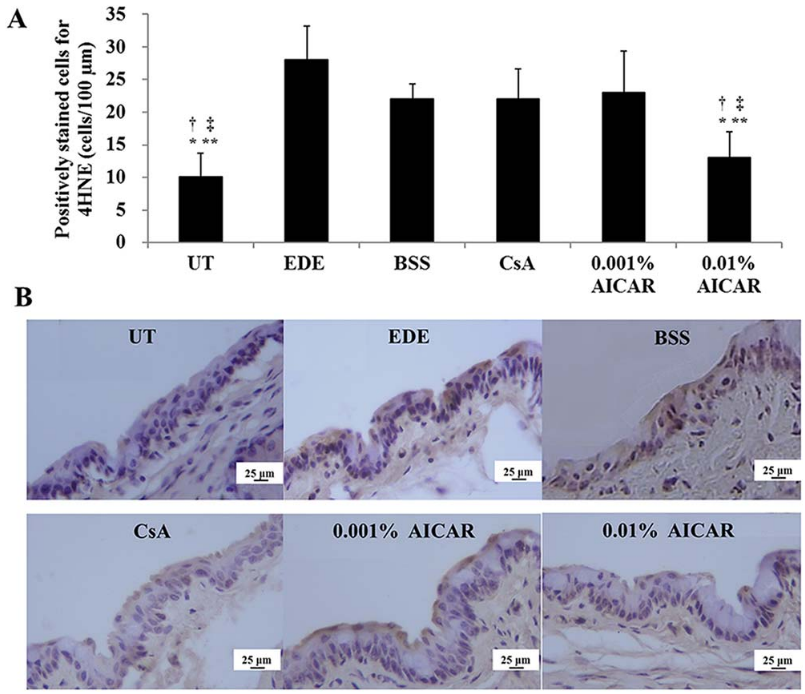

Figure 8. The number of cells positively stained for 4HNE in the conjunctiva (**A**) and immunohistochemical staining of representative specimens (**B**) in the UT, EDE control, BSS-treated, 0.05% CsA-treated, 0.001% AICAR-treated, and 0.01% AICAR-treated groups after 10 days of treatment. The 0.01% AICAR-treated group shows a significantly lower number of positively stained cells compared with the EDE, CsA, and 0.001% AICAR-treated groups. **P* < 0.05 compared with the EDE group. ***P* < 0.05 compared with the BSS group. †*P* < 0.05 compared with the CsA group. ‡*P* < 0.05 compared with the 0.001% AICAR group. *Scale bars*: 25 μm.

pathways, such as p38 mitogen-activation protein kinase (MAPK).[36] Similarly, AICAR activates AMPK and ameliorates proinflammatory activities in several cell types, including hepatocytes, adipocytes, smooth muscle cells, and various inflammatory cells.[14–16] Previous studies have demonstrated that AICAR controls the balance between CD4+ T helper cells and inducible regulatory T cells.[37,38] Another study has also shown that AICAR treatment led to reduction in TNF-α and IL-6 levels in alveolar macrophages and decreased lung inflammation in mice.[39] It has been reported that AMPK activation induced by AICAR can inhibit NF-κB activation and thus downregulates TNF production by macrophages. The effectiveness of AICAR was shown for certain inflammatory diseases in murine models, such as acute lung injury, autoimmune encephalomyelitis, and inflammatory bowel disease.[15,19,40]

In ophthalmology, Suzuki et al.[20,21] reported that systemic administration of AICAR inhibited NF-κB signaling through AMPK activation in the eye. As it is well known that dry eye stimulates MAPK activation, which leads to an increase in NF-κB activity and inflammatory mediators,[41,42] we studied the therapeutic effect of AMPK activation via the topical application of AICAR for treating the various ocular signs and reversing the inflammatory changes associated with dry eye. The therapeutic efficacy of 0.01% AICAR was comparable or superior to that of CsA, which is a well-known and effective treatment modality for DED.

Levels of p-AMPKα and p-NF-κB p65 are regarded as a marker of AMPK and NF-κB activation. In this study, we have demonstrated significant decline of AMPK activity and increase of NF-κB activity in the EDE mice. In contrast, the application of AICAR reversed AMPK deactivation and NF-κB activation in the conjunctival tissue. These findings are compatible with previous studies that AMPK activation induced by AICAR can inhibit NF-κB activation and suggest that anti-inflammatory effects of AICAR are mediated by the AMPK dependent pathway.

In the current study, we investigated the effect of AICAR on several well described measures of ocular surface inflammation: inflammatory cytokine and chemokine levels and percentages of CD4+ CXCR3+ T cells in the conjunctiva. Cyclosporine A was chosen as the comparative treatment because its therapeutic efficacy has been reported in many DED experiments. In accordance with the previously described results, we observed increased IL-1β, IL-6, TNF-α, IFN-γ, IP-10, and MIG concentrations in the EDE group.[4,6,25,29,28,36] After treatment with 0.01% AICAR, the levels of these cytokines and chemokines decreased and similar suppression was found in the CsA group; however, it was more prominent in the 0.01% AICAR group. In contrast, the 0.001% AICAR group did not show a significant reduction in these parameters. We hypothesize that the inhibition of NF-κB signaling by AICAR downregulated the expression of inflammatory molecules, such as IL-1β, IL-6, and TNF-α.

Interferon γ is a multifaceted cytokine that is essential for proper immune function. It is usually considered a proinflammatory cytokine produced by Th1 cells and natural killer cells that induces macrophage activation. Beyond the well documented and critical role of IFN-γ in host defense, it is known to induce goblet cell loss in DED via induction of conjunctival epithelial apoptosis.[43] In the present study, the 0.01% AICAR treatment significantly reduced the concentrations of IFN-γ, IP-10, and MIG in the conjunctiva, compared with the EDE, BSS, CsA, and 0.001% AICAR groups. In addition, as we expected, the mean goblet cell density was highest in the 0.001% AICAR group. These findings led us to speculate that AICAR might reduce IFN-γ and impair IFN-γ–induced gene expression including MIG and IP-10 via AMPK activation, which is consistent with a previous study that demonstrated that AMPK activation suppressed IFN-γ and CCL2 expression in the central nervous system.[44]

Homing and infiltrating T cells onto the ocular surface consists of predominantly CD4+ T cells in DED. Chemokine receptors that are Th1-related—such as CCR5 and CXCR3—and their ligands play an important role in the trafficking of activated CD4+ T cells.[6] We have previously found that desiccating stress stimulates the expression of inflammatory cytokines and Th-1 chemokines and their receptors, CCR5 and CXCR3, in EDE tear film and ocular surface.[6,25,28,29] In our study, we found that treatment with topical CsA or 0.01% AICAR significantly reduced the number of CD4+ CXCR3+ T cells in the conjunctiva.

The effects of chronic inflammation include induction of oxidative stress and apoptotic cell death, which can contribute to cell structure and functional abnormalities. It has been shown that oxidative stress is linked to corneal, conjunctival, and lacrimal gland injury that is associated with DED and lipid peroxide and myeloperoxidase activity increase in the tears of dry eye patients.[45,46] The therapeutic efficacy of AICAR is supported further by antioxidative effects through decreased staining for the oxidative stress damage marker 4HNE, which is one of the end products of lipid peroxidation. AMPK function as an early warning system in response to oxidants so as to attenuate oxidative injury. AMPK activation contributes to an increase in intracellular levels of NADPH, which is the major reducing equivalent in human cells. Disruption of AMPK activation under oxidative stress triggers cell death via accumulation of oxidative damage caused by reactive oxygen species. Awad et al.[47] previously identified that AICAR controlled oxidative stress resistance by increasing the expression of catalase, an antioxidant enzyme. Consistent with his finding, in the present study, there was a significant decrease in the number of 4HNE positive–stained cells in 0.01% AICAR treated mice, whereas no changes were found in CsA-treated mice.

We further investigated AICAR's effect on various clinical parameters, including tear volume, tear film BUT, and corneal fluorescein staining, in a mouse model of EDE. Despite continuous exposure to desiccating stress and rigorous anticholinergic treatment, both 0.01% AICAR-treated eyes and CsA-treated eyes showed increases in tear production and reversal of corneal epithelial damage as determined by a decrease in corneal fluorescein uptake. In addition, the 0.01% AICAR group showed better improvement in tear film BUT and corneal fluorescein staining than the CsA group. Goblet cells secrete mucin that contributes to tear film stability and AICAR might result in improvement of BUT. Furthermore, as Sano et al.[22] reported, AICAR might regulate the activity of epithelial sodium channels through changing the fluidity and surface charge of phospholipid membranes and increased tear secretion. It was also reported that AICAR increased saliva secretion which is mainly controlled by the autonomic nervous system, similar to tear secretion.[48]

Regarding the epithelial damage, the possible explanation about the mechanism of corneal staining improvement is as follows; First, TNF-α can play a role in corneal epithelial barrier function by inducing the expression of MMPs and loss of tight junctions from superficial corneal epithelial cells.[49,50] Suppression of TNF-α in the AICAR group might be associated with the improvement of corneal staining. Second, the superficial layer of the corneal epithelium, comprising a strong barrier, is very susceptible to tear deficiency.[51] Hence, the improvement of corneal staining in the present study might be attributed to increased tear volume in the AICAR group. Third, improvement of goblet cell density might induce an increase in mucin secretion and protected the ocular surface from desiccation and losses of corneal epithelial resistance.[52]

Taken into consideration, our findings as well as the results of the aforementioned studies, we suggest that topical AICAR ameliorates DED as determined by clinical, inflammatory, and oxidative measures. The therapeutic effect of 0.01% AICAR was comparable or superior to that of CsA. The results presented herein support the notion that AICAR has great potential as a therapeutic agent for the treatment of dry eye.

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