Bacillus-Derived Manganese Superoxide Dismutase Relieves Ocular-Surface Inflammation and Damage by Reducing Oxidative Stress and Apoptosis in Dry Eye

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Purpose. We hypothesized that antioxidative enzymes supplementation could be a treatment option for dry eye. We investigated the efficacy of oral administration of *Bacillus*-derived superoxide dismutase (Bd-SOD) in a murine experimental dry eye (EDE).

Methods. In part I, mice were randomly assigned to normal control, EDE, and mice groups that were treated with oral Bd-SOD after induction of EDE (EDE + Bd-SOD group; four mice in each group). Expression of SOD2, a major antioxidant enzyme with manganese as a cofactor, was assessed by immunofluorescence staining. In part II, mice were divided into seven groups (six mice in each group): normal control, EDE, vehicle-treated, topical 0.05% cyclosporin A (CsA)-treated, and oral Bd-SOD-treated (2.5, 5.0, and 10.0 mg/kg Bd-SOD) groups. Tear volume, tear-film break-up time (TBUT), and corneal fluorescein-staining scores (CFS) were measured at zero, five, and 10 days after treatment. Ten days after treatment, 2′,7′-dichlorodihydrofluorescein diacetate for reactive oxygen species (ROS), enzyme-linked immunosorbent for malondialdehyde, and TUNEL assays for corneal apoptosis, flow cytometry inflammatory T cells, and histological assessment were performed.

Results. Compared to the normal control group in part I, the EDE group showed significantly decreased SOD2 expression by immunofluorescence staining. However, the EDE + Bd-SOD group recovered similar to the normal control group. In part II, ROS, malondialdehyde, and corneal apoptosis were decreased in CsA and all Bd-SOD-treated groups. Corneal and conjunctival inflammatory T cells decreased, and conjunctival goblet cell density increased in CsA-treated and Bd-SOD–treated groups. Compared to the CsA-treated group, the 2.5 mg/kg Bd-SOD–treated group showed increased TBUT and decreased inflammatory T cells, and the 5.0 mg/kg Bd-SOD-treated group showed decreased CFS and increased conjunctival goblet cells.

Conclusions. Oral Bd-SOD administration might increase autogenous SOD2 expression in ocular surface tissue in EDE and could be developed as a complementary treatment for DE in the future.

Keywords: superoxide dismutase, oxidative stress, apoptosis, bacillus-derived, dry eye

A vicious circle of inflammation affects the ocular surface of dry eye (DE), causing sustained symptoms, aggravation of clinical severity, and neurological pain.[1,2] Oxidant-antioxidant imbalance is a pathological process that exacerbates inflammation through proinflammatory pathways.[3–6] In subjects with DE, the levels of protective antioxidants are decreased, and a vast reactive oxygen species (ROS) is generated in the corneal tissue.[2,6] An intense free-radical stress burden, such as that induced by particulate matter, fossil fuel exhaust, or solar ultraviolet radiation, could intensify this vicious cycle and aggravate DE.[6–8] Several previous studies have shown that topical or systemic administration of antioxidants in the form of nutrients, including lactoferrin, vitamins D or E, and catechin, and some medicinal plant extracts can alleviate DE symptoms and signs.[9–13]

The superoxide dismutase (SOD) enzyme family is a major and effective antioxidant system. It consists of three isoenzymes (SOD1 and SOD3, using copper/zinc as cofactors; and SOD2, using manganese [Mn] as a cofactor).[14,15] The accumulation of ROS because of deficiency of SOD in ocular tissues can lead to DE and meibomian gland dysfunction.[3,5,16] Several studies have shown that SOD1-deficient mice exhibit morphological and secretory functional changes in the lacrimal and meibomian glands, leading to DE.[5,17–19] In addition, topical treatment with a SOD mimetic, Mn(III) tetrakis(1-methyl-4-pyridyl) porphyrin, showed similar or slightly superior efficacy to 0.05% topical cyclosporine (CsA) in improving signs of experimental dry eye (EDE) in vivo, by reducing oxidative stress in the corneal epithelium.[20]

Mn-SOD scavenges mitochondrial ROS and protects against cell death.[14,15] Consequently, this protein plays an anti-apoptotic role against oxidative stress and inflammatory cytokines.[21,22] Dietary Mn-SOD of *Bacillus amyloliquefaciens* (GF423) have been reported to reduce oxidative stress in γ-radiation- and DSS-induced colitis mouse models and suggested to be useful in prevention or treatment of ulcerative colitis.[23–25] In these studies, oral administration of *Bacillus*-SOD increased activity of antioxidant enzymes such as SOD, glutathione peroxidase, and catalase in the blood. We hypothesized that supplementation of deficient enzymes in ocular tissues is a promising treatment option for DE to prevent the side effects associated with the long-term and frequent use of multiple eye drops. Thus this study investigated whether the oral administration of Mn-SOD affected the expression of SOD2 in ocular surface tissues in a murine model of DE. Furthermore, we investigated the efficacy of oral administration of *Bacillus*-derived-Mn-SOD (Bd-SOD; GF103) on the clinical signs, oxidative damage, and inflammation in a murine model of EDE.

Material and Methods

GF103 is a mutant manganese superoxide dismutase of Bacillus velezensis (Bacillus amyloliquefaciens GF423), which consists of 200 amino acids and forms a dimer of 44.5 kDa (see Fig. 7).[26] It is manufactured by GenoFocus, Inc. (Daejeon, South Korea) and was provided by BiomLogic, Inc. (Seoul, South Korea). Bd-SOD (GF103) was coated with Shellac (Excelacs Co., Ltd., Bangkok, Thailand) to protect it from gastric acid. In brief, Shellac was dissolved in 3% ethanol, sterilized using a 0.2-µm microfilter, and diluted with PBS. Shellac and Bd-SOD (GF103) solutions (20 mg/mL) were mixed in a 1:1 ratio and lyophilized. The lyophilized shellac-coated Bd-SOD was mixed with maltodextrin (Daesang Co., Ltd., Seoul, South Korea) in a ratio of 1:9–12 to prepare the material used for testing. The SOD activity of the Shellac-coated Bd-SOD (GF103) was set to 90–110 U/mg.

All the animals were treated in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. In this study, female C57BL/6 mice aged six to eight weeks were used. The mouse model of EDE was induced by exposure to an air draft and subcutaneous injection of scopolamine three times a day for 15 days, as previously described.[27]

We conducted two parts of experiments, each consisting of three repetitions using independent mice. In part I, the mice were randomly assigned to three groups (n = 4 per group) as follows: (1) normal control mice (normal control group); (2) EDE control mice that received no treatment other than induction of EDE (EDE group); and (3) mice that were treated with oral administration of Bd-SOD (5.0 mg/kg) after induction of EDE (EDE + Bd-SOD group). In part II, the mice were randomly assigned to seven groups (n = 6 per group) based on treatment, as follows: (1) normal control group; (2) EDE group; (3) vehicle control mice (vehicle group), treated with PBS; (4) topical CsA mice (topical CsA group), treated with topical 0.05% CsA; (5) 2.5 mg/kg Bd-SOD-treated mice (2.5 mg/kg Bd-SOD group); (6) 5.0 mg/kg Bd-SOD-treated mice (5.0 mg/kg Bd-SOD group); and (7) 10.0 mg/kg Bd-SOD-treated mice (10.0 mg/kg Bd-SOD group).

In topical CsA groups, eye drops (2 µL) were applied topically, twice a day for 10 days. In the Bd-SOD-treated groups, Bd-SOD powder was dissolved in PBS at the indicated concentration, and orally administered by gavage once daily for 10 days. In the vehicle group, PBS was orally administered once a day for 10 days. In part I, immunofluorescence staining was performed to identify SOD2 expression after euthanization. In part II, clinical parameters, including tear volume, tear film break-up time (TBUT), and corneal fluorescein-staining (CFS), were measured at baseline and at five and 10 days after treatment. After measuring the clinical parameters, the mice were euthanized, and a 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA) assay for ROS, ELISA for detecting malondialdehyde (MDA) as oxidative stress, TUNEL assay for corneal apopotosis, flow cytometry for inflammatory T-cells, and histological analysis were performed.

Immunofluorescence staining was performed on cryosections of the conjunctiva and lacrimal gland tissues of the harvested eyeball and adnexa. Sections were incubated at 4°C overnight with mouse monoclonal anti-SOD2 antibody (1:50 dilution; Santa Cruz, Dallas, TX, USA). Sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Vector, Burlingame, CA, USA) for 5 min. Cell images were obtained with following settings: excitation at 405 and 488 nm and emission at 424–472 nm and 502–550 nm for SOD2 expression and DAPI, respectively. The results were expressed as the average number of positively stained cells.

Tear volume was measured using phenol red cotton threads (Zone-Quick; Oasis, Glendora, CA, USA).[28] Tear uptake distances measured by threads converted to tear volumes using previously calculated standard curve.[28] Sodium fluorescein (1%; 1 µL) was instilled into the inferior conjunctival sac. After three blinks, TBUT was recorded in seconds under slit-lamp biomicroscopy (BQ-900; Haag-Streit Bern, Switzerland) with cobalt blue light. After 90 seconds, punctate staining of the cornea was evaluated. CFS was calculated using a four-point scale per quadrants as previous study (maximum 16 points).[29]

Extracellular ROS levels in the corneal, conjunctival, and lacrimal gland tissues were measured using a CM-H2DCFDA kit with the FACSCalibur flow cytometer (BD Biosciences, CA, USA) at an excitation wavelength of 480 nm and an emission wavelength of 530 nm.[30] The results are expressed as the percentage relative to the normal control group, using CellQuest software (version 5.2.1; BD Biosciences). Total MDA levels were detected using ELISA (Quantikine, R&D Systems, Minneapolis, MN, USA). Detection of MDA protein in the corneal, conjunctival, and lacrimal gland tissues was performed three times using a commercial sandwich ELISA kit with capture and detection antibodies. A TUNEL assay was used to detect the 3′-hydroxyl ends of fragmented DNA to identify apoptotic cells in the corneal tissues. Staining was evaluated using the DeadEnd Fluorometric TUNEL System (Promega Corp., Madison, WI, USA). Cell images were obtained separately with excitation wavelengths at 405 and 488 nm and emission wavelengths at 424 to 472 and 502 to 50 nm for the TUNEL assay and DAPI staining, respectively. TUNEL-positive cells and DAPI staining of cell nuclei were perceived under a microscope with 20-fold magnification.

Flow cytometry was performed to examine the number of $CD4^+$ IFN-γ^+ T cells in the cornea and conjunctiva.[31] Samples were incubated with fluorescein-conjugated anti-CD4 antibody (BD Biosciences), phycoerythrin-conjugated anti-IFN-γ antibody (BD Biosciences), and isotype control antibody at 37°C for 30 minutes. The number of cells was counted using a FACSCalibur cytometer with Cell Quest

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software (BD Biosciences). For periodic acid–Schiff (PAS) staining, serial sections (6-µm-thick) were cut and stained with 0.5% PAS to identify goblet cells. Sections from each mouse group were take pictures using a microscope with a digital camera.

Statistical analysis software (SPSS version 22.0; SPSS Inc., Chicago, IL, USA) was used. The normal distribution of the data was confirmed using the Shapiro-Wilk test. Statistical differences among the groups were determined by repeated-measure analysis of variance tests followed by Dunnett's post hoc tests or the Kruskal-Wallis test with Fisher's least significant difference post hoc analysis.

Results

The mean SOD2-positive cell counts using immunofluorescence staining in the conjunctival epithelium were 16.3 ± 1.25 cells/100 µm in the normal control group, 2.67 ± 0.94 cells/100 µm in the EDE, and 14.7 ± 1.25 cells/100 µm in the EDE + Bd-SOD groups in part I of the study (Fig. 1b). The mean SOD2-positive cell counts in the lacrimal gland were 20.3 ± 2.05 cells/100 µm in the normal control, 8.3 ± 1.25 cells/100 µm in the EDE, and 22.3 ± 1.25 cells/100 µm in the EDE + Bd-SOD groups, respectively (Fig. 1c). Mice in the EDE group had significantly lower expression of SOD2 in the conjunctival and lacrimal gland tissue than did those in the normal control group ($P < 0.01$ and $P = 0.03$). There were no significant differences in SOD2 expression between the normal control and EDE + Bd-SOD groups in the conjunctiva or lacrimal glands. Bd-SOD treatment could restore the SOD2 expression in the conjunctiva and lacrimal glands compared to that in the normal control group.

In part II, the mean tear volume was 0.045 ± 0.006 µL in the normal control, 0.02 ± 0.003 µL in the EDE, 0.021 ± 0.003 µL in the vehicle, 0.026 ± 0.004 µL in the topical CsA, 0.026 ± 0.005 µL in the 2.5 mg/kg Bd-SOD, 0.026 ± 0.005 µL in the 5.0 mg/kg Bd-SOD group, and 0.026 ± 0.005 µL in the 10.0 mg/kg Bd-SOD group. Ten days after treatment, the mean tear volume was 0.043 ± 0.005 µL (normal control), 0.017 ± 0.003 µL (EDE), 0.018 ± 0.003 µL (Vehicle), 0.027 ± 0.005 µL (topical CsA), 0.027 ± 0.005 µL (2.5 mg/kg Bd-SOD), 0.03 ± 0.005 µL (5.0 mg/kg Bd-SOD), and 0.028 ± 0.004 µL (10.0 mg/kg Bd-SOD). All treatment groups showed a significant increase in tear volume as compared with the EDE group at 5 and 10 days (all $P < 0.01$). Furthermore, the 5.0 mg/kg Bd-SOD group showed a significant increase in tear volume as compared with the topical CsA group (Fig. 2a).

The values of TBUT were 2.21 ± 0.40 s (normal control), 1.02 ± 0.20 s (EDE), 1.06 ± 0.23 s (Vehicle), 1.3 ± 0.20 s (topical CsA), 1.23 ± 0.20 s (2.5 mg/kg Bd-SOD), 1.31 ± 0.30 s (5.0 mg/kg Bd-SOD), and 1.28 ± 0.30 s (10.0 mg/kg Bd-SOD) at 5 days. At 10 days, the values for TBUT were 2.1 ± 0.40 s (normal control), 0.91 ± 0.20 s (EDE), 0.93 ± 0.20 s (Vehicle), 1.39 ± 0.20 s (topical CsA), 1.21 ± 0.30 s (2.5 mg/kg Bd-SOD), 1.28 ± 0.30 s (5.0 mg/kg Bd-SOD), and 1.3 ± 0.30 s (10.0 mg/kg Bd-SOD). All treatment groups showed a significant increase in TBUT compared with the EDE and vehicle groups (all $P < 0.01$) at five and 10 days. In addition, the 2.5 mg/kg Bd-SOD group showed a significant increase in TBUT as compared to the topical CsA group ($P < 0.01$; Fig. 2b).

At five days, the CFS in the normal control, EDE, vehicle, topical CsA, 2.5mg/kg, 5.0 mg/kg, and 10.0 mg/kg Bd-SOD were 2.9 ± 1.4, 12.1 ± 2.1, 11.7 ± 2.2, 10.4 ± 2.5, 10.4 ± 1.9, 9.9 ± 2.5, and 10.1 ± 2.9, respectively. At 10 days, the respective CFS was 3.6 ± 1.4 in the normal control, 14.1 ± 1.8 in the EDE, 13.5 ± 1.6 in the vehicle, 8.9 ± 2.0 in the topical CsA, 8.8 ± 2.0 in the 2.5 mg/kg Bd-SOD, 7.8 ± 1.9 in the 5.0 mg/kg Bd-SOD, and 8.2 ± 1.9 in the 10.0 mg/kg Bd-SOD groups. All treatment groups showed a significant decrease in CFS as compared with the EDE and vehicle groups at 5 and 10 days (all $P < 0.01$). In addition, the 5.0 mg/kg Bd-SOD group had significantly decreased CFS as compared to the topical CsA group at 10 days (Fig. 2c).

Compared to the EDE and vehicle groups, a significantly lower intensity of DCF-DA was observed in the cornea, conjunctiva, and lacrimal gland in all treatment groups (all $P < 0.05$). In addition, the 2.5 mg/kg Bd-SOD group showed a significant decrease in ROS levels as compared to the topical CsA group in the cornea (Fig. 3; $P = 0.04$).

The Table shows the MDA levels in response to oxidative stress. The MDA levels in the cornea, conjunctiva, and lacrimal gland in all treatment groups were significantly lower than those in the EDE and vehicle groups (all $P < 0.05$). There was no significant difference in MDA levels among the different treatment groups.

The mean apoptotic cell counts in the cornea were 1.6 ± 0.5 cells/100 µm, 14.8 ± 3.3 cells/100 µm, 2.1 ± 3.0 cells/100 µm, 6.1 ± 2.1 cells/100 µm, 6.2 ± 1.1 cells/100 µm, 4.0 ± 1.3 cells/100 µm, and 4.6 ± 1.3 cells/100 µm in the normal control, EDE, vehicle, topical CsA, and 2.5 mg/kg, 5.0 mg/kg, and 10.0 mg/kg Bd-SOD groups, respectively (Fig. 4b). Apoptotic cell counts in the corneal epithelium were significantly lower in all treatment groups than those in the EDE and vehicle groups (all $P < 0.01$).

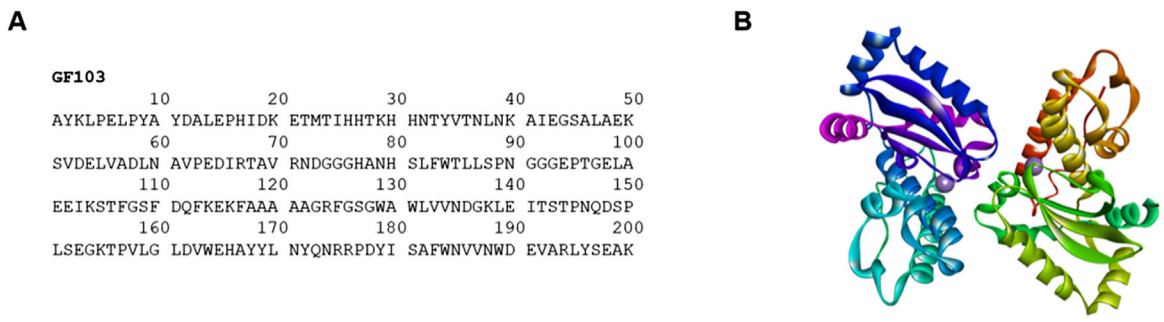

Figure 1. Amino acid sequence of GF103 (**A**). Predicted structure of GF103 using SWISS-MODEL server. Manganese are represented as purple CPK balls (**B**).

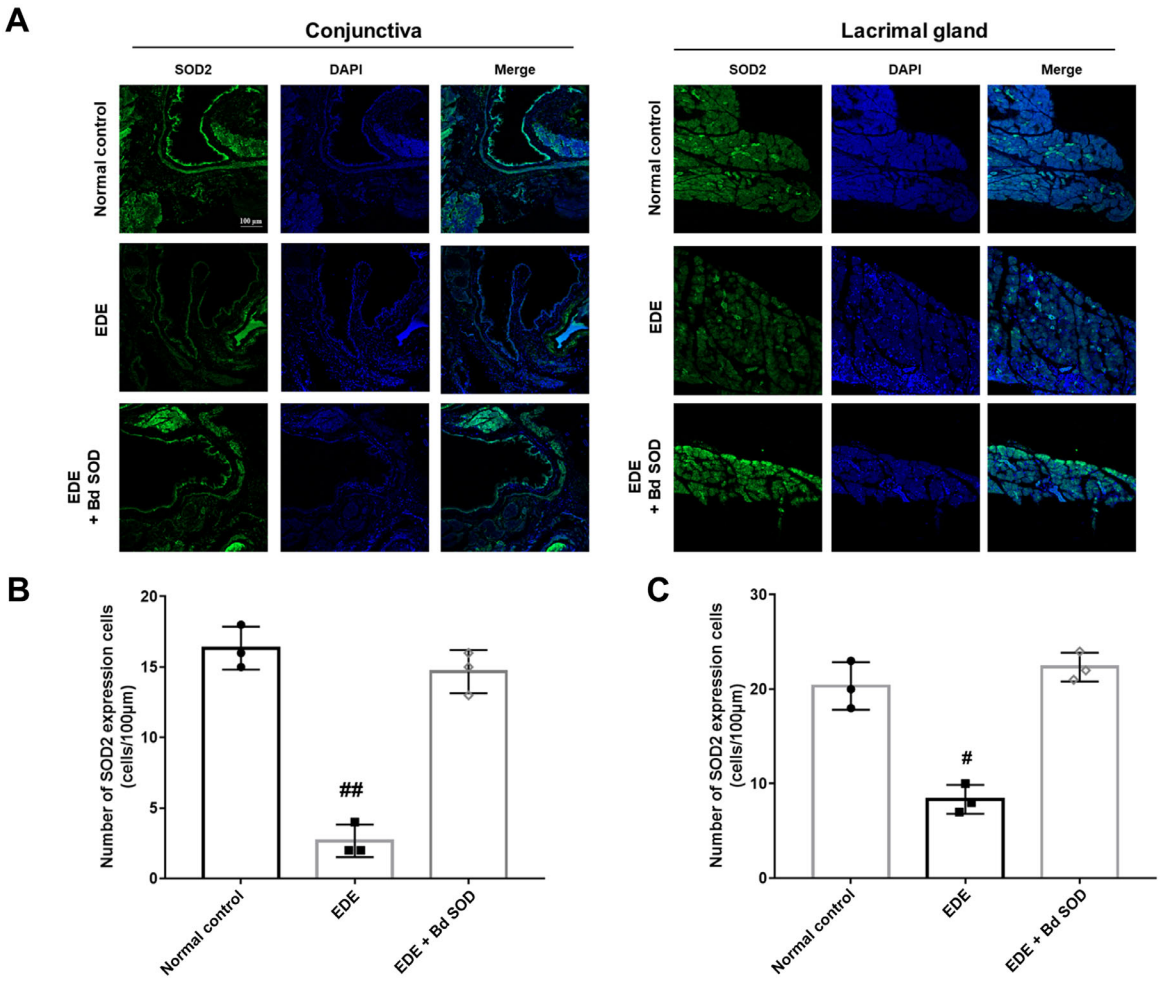

Figure 2. Representative photograph of immunofluorescent staining (**A**) and the mean number of SOD2-expressing cells in conjunctival (**B**) and lacrimal (**C**) tissues of the normal control, experimental dry eye (EDE), and EDE+ *Bacillus*-derived SOD (Bd-SOD) groups at 10 days. $^{\#}P < 0.05$ versus normal control; $^{\#\#}P < 0.01$ versus normal control.

In the cornea and conjunctiva, the $CD4^+$ IFN-γ^+ T cell counts were 16.53% ± 1.65% and 17.40% ± 3.43% (normal control), 48.39% ± 1.34% and 46.63% ± 1.27% (EDE), 47.02% ± 0.04% and 43.65% ± 2.68% (vehicle), 26.05% ± 0.18% and 22.67% ± 0.47% (topical CsA), 21.18% ± 0.15% and 21.00% ± 0.63% (2.5 mg/kg Bd-SOD), 23.87% ± 0.17% and 22.87% ± 2.47% (5.0 mg/kg Bd-SOD), and 30.24% ± 1.33% and 25.83% ± 0.27% (10.0 mg/kg Bd-SOD). The numbers of CD4+ IFN-γ+ T cells in the cornea and conjunctiva were lower in all treatment groups than those in the EDE and vehicle groups (all $P < 0.01$). In addition, the percentage of corneal inflammatory T cells in the 2.5 mg/kg Bd-SOD group was significantly lower than that in the topical CsA group (Fig. 5; $P = 0.01$).

The mean goblet cell counts were 52.1 ± 9.6 cells/100 μm (normal control), 14.1 ± 3.6 cells/100 μm (EDE), 14.6 ± 2.4 cells/100 μm (vehicle), 25.2 ± 3.4 cells/100 μm (topical CsA), 27.9 ± 6.9 cells/100 μm (2.5 mg/kg Bd-SOD), 35.8 ± 10.2 cells/100 μm (5.0 mg/kg Bd-SOD), and 32.2 ± 3.9 cells/100 μm (10.0 mg/kg Bd-SOD). All treatment groups showed a higher conjunctival goblet cell density than those in the EDE and vehicle groups (all $P < 0.05$). The goblet cell density in the 5 mg/kg Bd-SOD group was higher than that in the topical CsA group ($P = 0.01$; Fig. 6b).

Discussion

Intracellular ROS levels are regulated by the balance between ROS-generating system and antioxidant enzymes, including SOD, catalase, glutathione peroxidase, and the thioredoxin system.[14] SOD is ubiquitous in all aerobic organisms, and it catalyzes the conversion of O_2 to H_2O_2 and O_2.[14,32] It has been suggested that enhanced SOD activity could upregulate other antioxidant enzymes by increasing the endogenous antioxidant mechanism.[32,33]

SOD is a protein with high-molecular-weight, it is difficult to absorb intact SOD from the gastrointestinal tract after oral administration.[32] Moreover, the isolated SOD enzyme is rendered chemically inactive and accordingly ineffective because of the low pH and high proteolytic enzyme of the gastrointestine.[32] To solve these bioavailability issues, several groups have designed various coatings using lipids or proteins to encapsulate SODs.[33,34] Nongenetically modified cantaloupe melon (*Cucumis melo* LC) were used as a source of this enzyme previously because this plant produces high levels of SOD (100 U/mg).[32] Several studies have demonstrated the effect of coated-melon SOD in ROS-related diseases, such as cancer, cardiovascular disease, degenerative diseases, and infectious diseases.[33,35–37] These studies have supported the oral administration

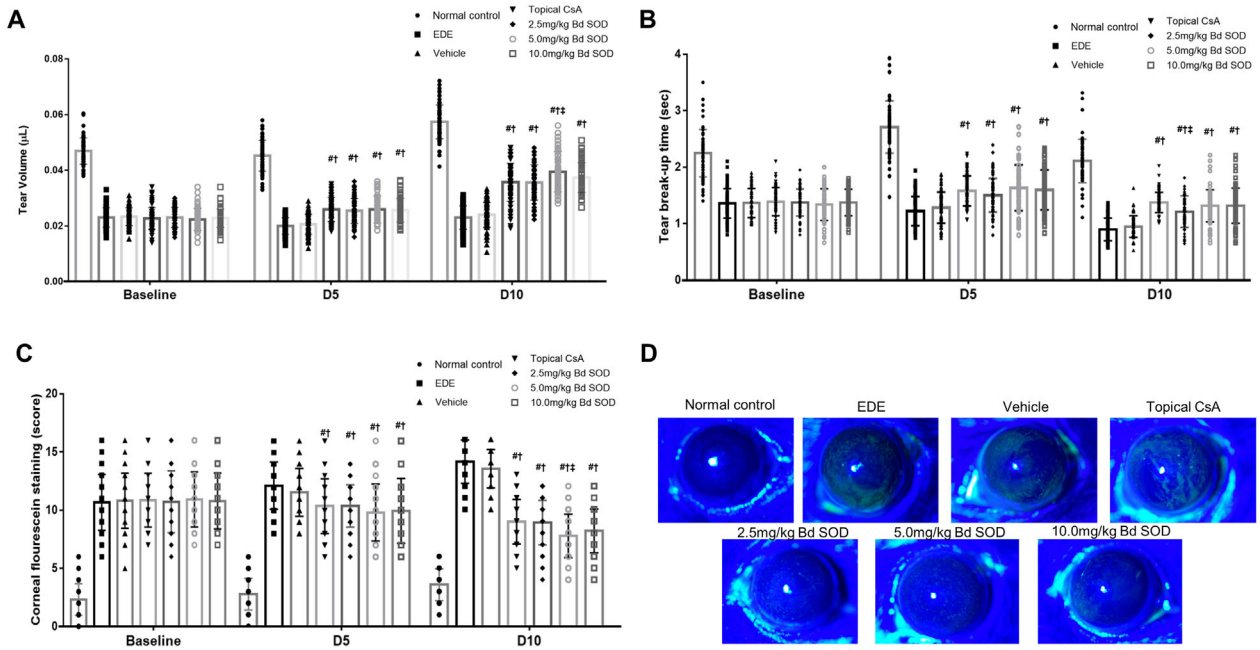

Figure 3. Mean tear volumes (**A**), tear-film break-up time (**B**), corneal fluorescein-staining scores (**C**), and representative photographs (**D**) of the normal control, EDE, vehicle, topical 0.05% CsA, 2.5 mg/kg Bd-SOD, 5.0 mg/kg Bd-SOD, and 10.0 mg/kg Bd-SOD-treated groups at baseline, day five, and day 10. #$P < 0.05$ versus EDE; †$P < 0.05$ versus vehicle; ‡$P < 0.05$ versus topical CsA.

Table. Malondialdehyde Levels in Cornea, Conjunctiva, Lacrimal Gland of the Normal Control, EDE, Vehicle, Topical 0.05% CsA, 2.5 mg/kg Bd-SOD, 5.0 mg/kg Bd-SOD, and 10.0 mg/kg Bd-SOD–Treated Groups

Groups	Cornea (pmol/mL)	Conjunctiva (pmol/mL)	Lacrimal Gland (pmol/mL)
Normal control	12.1 ± 3.16	17.8 ± 12.2	14.6 ± 7.25
EDE	30.9 ± 21.1	28.0 ± 8.92	31.0 ± 13.6
Vehicle	27.7 ± 27.4	26.1 ± 4.64	37.0 ± 12.6
Topical CsA	17.7 ± 9.62*,†	19.1 ± 7.99*,†	22.2 ± 11.4*,†
2.5 mg/kg Bd-SOD	22.7 ± 13.8*,†	21.5 ± 9.26*,†	22.6 ± 7.72*,†
5.0 mg/kg Bd-SOD	20.2 ± 14.0*,†	19.0 ± 7.17*,†	18.8 ± 11.6*,†
10.0 mg/kg Bd-SOD	18.5 ± 10.6*,†	16.3 ± 9.12*,†	22.5 ± 11.4*,†

Data are expressed as mean ± standard deviation.
* $P < 0.05$, compared with the EDE control group.
† $P < 0.05$, compared with the vehicle group.

of SOD as a complementary treatment for systemic diseases.[32,33,35–37]

Previous studies showed the effect of Mn-SOD extracted from *B. amyloliquefaciens* in a model of inflammatory bowel disease.[23–25] The production of Bd-SOD could be more economical, since microbial suspension culture is a more convenient method for large-scale production by mass cultivation of cells than plant cultivation.[23] There are many beneficial effects associated with increased SOD levels in animals or humans after oral administration of bacilli, which has become the basis for attempting SOD extraction from *Bacillus* spp.[38–41] In previous studies, Bd-SOD administration increased antioxidant enzyme activity, including that of SOD, catalase, and glutathione peroxidase, in the blood.[23,25] In addition, the rescue of SOD activity in SOD1-knockout mice by oral supplementation with Bd-SOD significantly ameliorated dextran sulfate sodium-induced colitis by suppressing p38 mitogen-activated protein kinase (MAPK)/nuclear factor-κB (NF-κB) signaling.[25]

In DE, desquamation of the corneal epithelium promotes increased osmolarity and inflammation, abnormal differentiation, apoptosis, and accelerated epithelial cell loss, with early activation of the MAPK/NF-κB pathways.[2,42] Various studies have suggested that oxidative stress is a major process exacerbating inflammation, a major mechanism in DE, via proinflammatory pathways.[3–6] Additionally, oxidative stress in the mitochondria at the lacrimal gland can induce lacrimal dysfunction.[43] Several previous studies have showed that topical or systemic application of antioxidant agents exerted positive effects on ocular surface and tear-film in DE.[9–13] Grumetto et al.[44] showed that the administration of the Mn-SOD gel formulation before ultraviolet radiation exposure could significantly reduce damage in rabbit ocular tissues. In another study, topical treatment with SOD mimetics showed similar or slightly superior results to

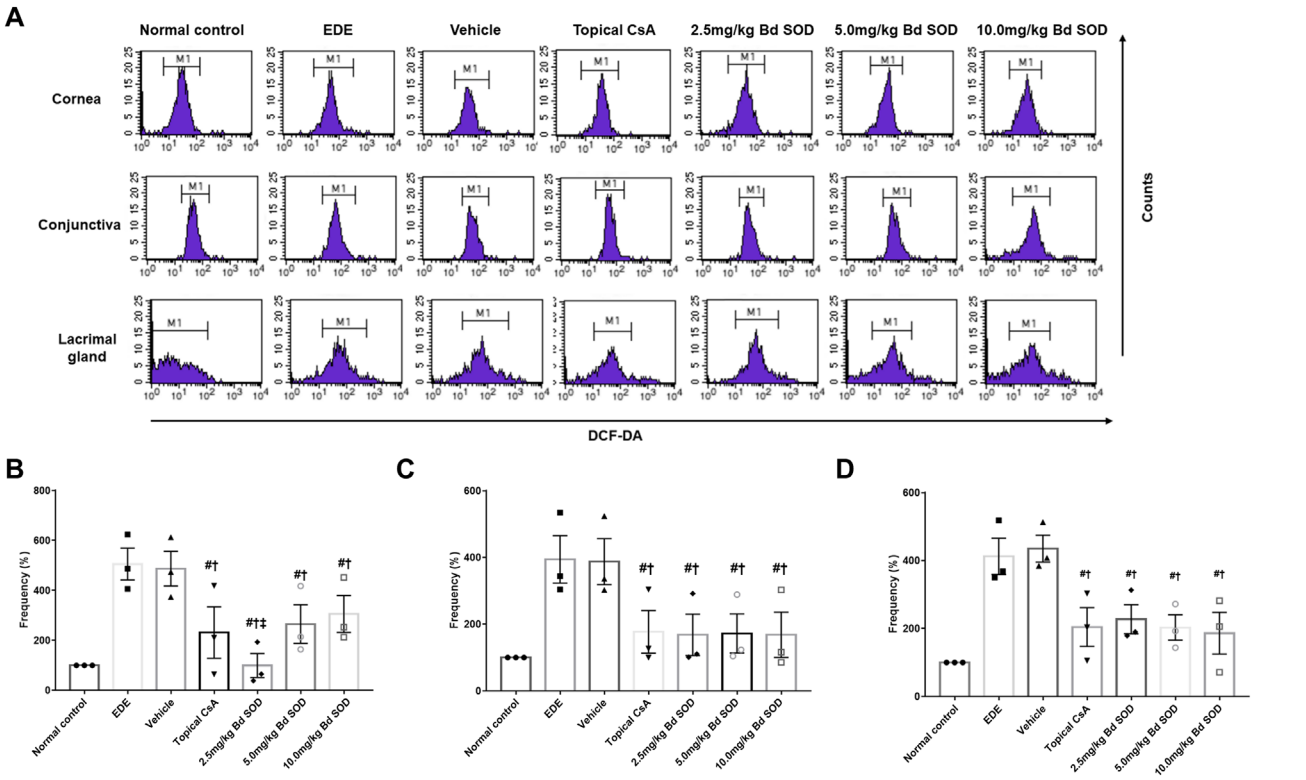

Figure 4. Levels of extracellular ROS measured using 2′,7′- DCF-DA in representative images (**A**), and frequency of DCF-DA in the cornea (**B**), conjunctiva (**C**), and lacrimal gland (**D**) of the normal control, EDE, vehicle, topical 0.05% CsA, 2.5 mg/kg Bd-SOD, 5.0 mg/kg Bd-SOD, and 10.0 mg/kg Bd-SOD-treated groups at day 10. #$P < 0.05$ versus EDE; †$P < 0.05$ versus vehicle; ‡$P < 0.05$ versus topical CsA.

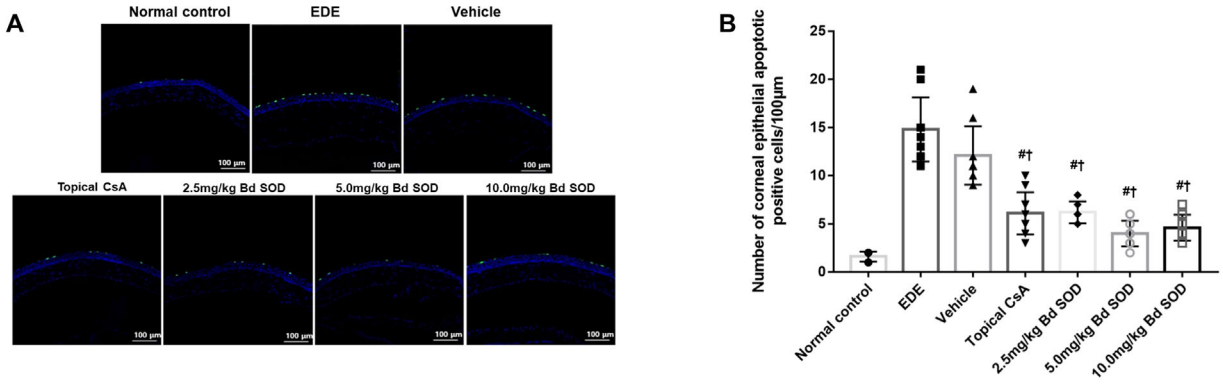

Figure 5. TUNEL staining in a representative photograph (**A**) and the mean number of TUNEL-positive cells (**B**) in the cornea of the normal control, EDE, vehicle, topical 0.05% CsA, 2.5 mg/kg Bd-SOD, 5.0 mg/kg Bd-SOD, and 10.0 mg/kg Bd-SOD-treated groups at day 10. #$P < 0.05$ versus EDE; †$P < 0.05$ versus vehicle; ‡$P < 0.05$ versus topical CsA.

topical treatment with CsA, in terms of several parameters, by direct permeation of corneal epithelial cells.[20] Therefore we hypothesized that supplementation of the insufficient SOD levels may be effective in the treatment of DE.

This study was planned based on the results of the previous study in which SOD and other antioxidant enzymes in the blood were increased by oral administration of Bd-SOD.[23,25] Therefore we investigated the expression of SOD2 in the conjunctiva and lacrimal gland, which are vascularized tissues, in assessing the therapeutic effect of Bd-SOD in EDE. In part I of our study, the EDE group showed significantly decreased expression of SOD2 in the conjunctival and lacrimal glands as compared to the normal control group. However, the SOD2 levels in the EDE + Bd-SOD group recovered to a level similar to that in the normal control control group. In part II of this study, all Bd-SOD treatment groups showed improvements in clinical parameters and inflammatory factors, as did the CsA-treatment group. ROS production, oxidative stress, and corneal apoptosis also decreased in the CsA group and in all three groups treated with different concentrations of Bd-SOD. In addition,

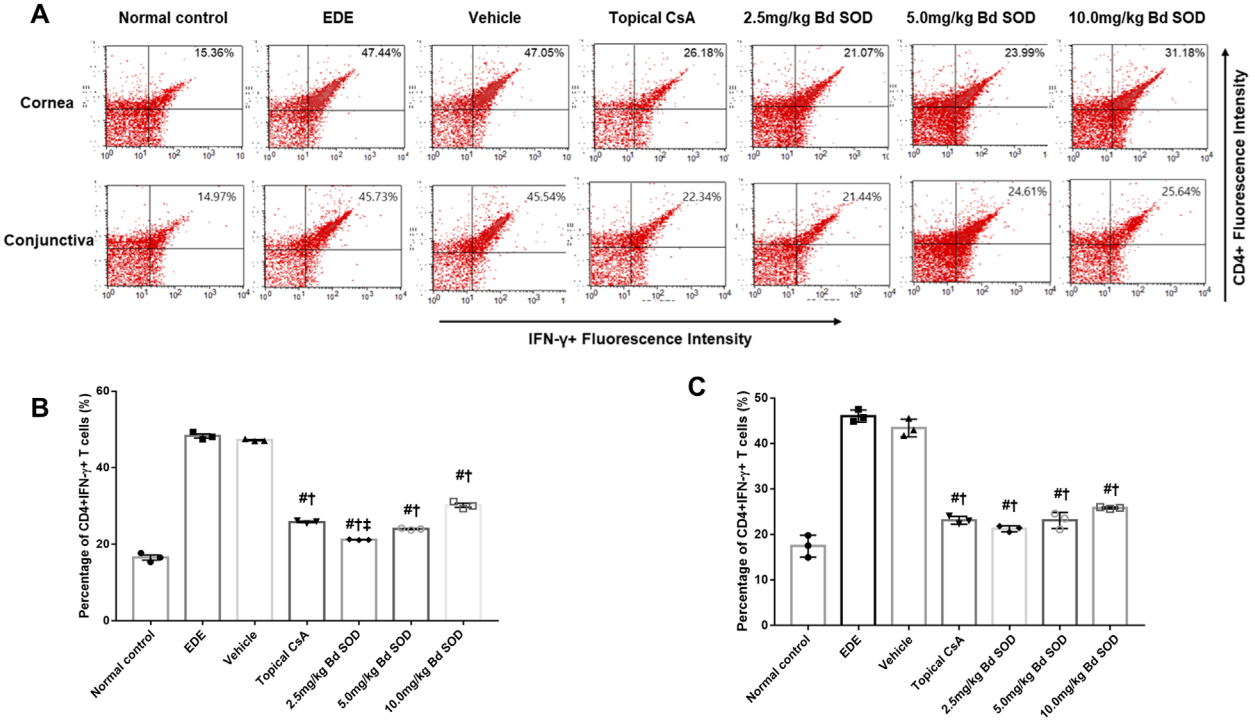

Figure 6. Flow cytometry showing $CD4^+IFN\text{-}\gamma^+$ T cells in (**A**) representative images, and the mean percentages of $CD4^+IFN\text{-}\gamma^+$ T cells in the (**B**) cornea and (**C**) conjunctiva of the normal control, EDE, vehicle, topical 0.05% CsA, 2.5 mg/kg Bd-SOD, 5.0 mg/kg Bd-SOD, and 10.0 mg/kg Bd-SOD-treated groups at day 10. $^{\#}P < 0.05$ versus EDE; $^{\dagger}P < 0.05$ versus vehicle; $^{\ddagger}P < 0.05$ versus topical CsA.

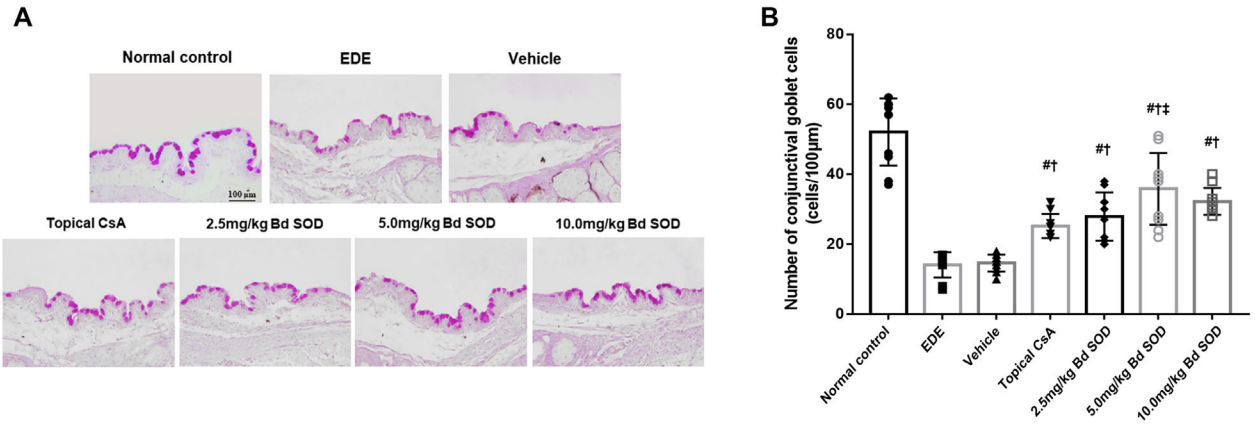

Figure 7. Periodic acid–Schiff staining of (**A**) representative specimens and (**B**) mean conjunctival goblet cell densities in the normal control, EDE, vehicle, topical 0.05% CsA, 2.5 mg/kg Bd-SOD, 5.0 mg/kg Bd-SOD, and 10.0 mg/kg Bd-SOD-treated groups at day 10. $^{\#}P < 0.05$ versus EDE; $^{\dagger}P < 0.05$ versus vehicle; $^{\ddagger}P < 0.05$ versus topical CsA.

some clinical and inflammatory parameters showed mildly superior efficacy with the Bd-SOD treatment as compared to the topical CsA treatment in our study. Compared to the CsA-treated group, the 2.5 mg/kg Bd-SOD–treated group showed higher TBUT and lower inflammatory T cells, and the 5.0 mg/kg Bd-SOD–treated group showed lower CFS and higher conjunctival goblet cells. The drug at a concentration of 10.0 mg/kg had no superior effect compared to 2.5 mg/kg or 5.0 mg/kg of Bd-SOD. Higher concentrations of the drug have the potential for increased toxicity, and this should be considered in future drug development. The two concentrations of Bd-SOD (2.5 mg/kg and 5.0 mg/kg) can be target concentrations for drug development and can be determined after application in humans.

Several studies have revealed that oral supplementation with coated SOD can increase endogenous antioxidant defences.[23,33,35,36,45] However, no experimental data that elucidate the detailed mechanism that oral supplementation of coated SODs induces systemic effects have been presented to date.[32] One study suggested that the systemic effects reported after SOD intake result from a cascade of events triggered in the small intestine, depending on the transcription factors via the antioxidant response element (ARE)/nuclear factor E2-related factor(Nrf2) axis.[32,46] Others

have hypothesized that nitric oxide (NO) might be produced in the gut in response to non-self-SOD signals, which then diffuses freely through tissues and causes release of autogenous SOD into the blood.[32,36] Some evidence supports the relationship between ARE/Nrf2 and NO mechanisms, suggesting that NO may regulate the expression of antioxidant genes by engaging the ARE/Nrf2 axis.[47–49]

This study is the first study on the oral administration of antioxidant enzymes in DE. In conclusion, our findings suggest that oral Bd-SOD administration might increase autogenous SOD2 expression in ocular surface tissue in a murine EDE model. Oral administration of Bd-SOD could improve oxidative damage and inflammation, resulting in improved clinical parameters in these EDE model mice. Our results suggest that Bd-SOD (GF103) could be developed as a complementary treatment for DE in the future.

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