APX-115A, a pan-NADPH oxidase inhibitor, reduces the degree and incidence rate of dry eye in the STZ-induced diabetic rat model

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Abstract. Dye eye disease (DED) is a common ocular disorder in patients with diabetes. It has been reported that APX-115A, a pan-nicotinamide adenine dinucleotide phosphate (NAPDH) oxidase inhibitor, has an apoptosis-inducing effect on Epstein-Barr virus-infected retinal epithelial cells, but its effects in DED are poorly understood. Therefore, a rat model of diabetes was used in the present study to investigate whether APX-115A has an impact on DED in diabetic rats. A diabetic model was established in male Sprague Dawley rats via the intraperitoneal injection of streptozotocin. The eyeballs of the rats were treated with a solution containing APX-115A or a saline control. Tear secretion was measured with the phenol red thread tear test, and the morphology of the eyeball and lacrimal gland tissues was determined using hematoxylin and eosin staining. In addition, localization of NAPDH oxidase 2 (NOX2) in the eyeball and lacrimal gland tissues was detected by immunohistochemistry. The APX-115A treatment had no effect on body weight, blood glucose level or the size of the lacrimal glands. However, morphological changes, namely intracellular vacuoles and acinar atrophy, were observed in the lacrimal glands of the diabetic rats, and APX-115A treatment attenuated these changes. Immunohistochemistry revealed that NOX2 expression was decreased in the lacrimal glands of the diabetic rats, and APX-115A treatment did not attenuate the reduction in NOX2. The corneas of the diabetic rats treated with APX-115A exhibited no change in thickness but had lower NOX2 expression levels compared with those of the control diabetic rats. APX-115A also increased tear secretion and ameliorated the histological changes associated with diabetes. Furthermore, the NOX2 expression levels in the corneas of the diabetic rats treated with APX-115A were restored to the levels observed in normal rats. These findings suggest that APX-115A has potential as a therapeutic agent for DED.

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Introduction

Diabetes mellitus (DM) is a common disease worldwide (1). According to the 10th edition of the IDF Diabetes Atlas published in 2021, >537 million adults aged 20-79 years are living with diabetes and a continuous increase in DM prevalence is predicted (2). This disease is characterized by uncontrolled and elevated blood glucose levels due to inadequate insulin secretion by the β cells of the pancreatic islets of Langerhans (3).

The pancreas is an organ in mammals that is comprised of α and β cells, and DM is associated with dysfunction of the β cells (3). DM can be categorized into type 1 diabetes (T1DM) and type 2 diabetes (T2DM) (3-5). T1DM is an autoimmune disorder that accounts for 5-10% of cases of diabetes and involves the destruction of β cells in the pancreas (5). This destruction results in a deficiency of insulin, a peptide hormone, which leads to insufficient glucose uptake by cells and increased blood glucose levels (6,7). T2DM is a progressive metabolic disorder that involves insulin resistance (8-10). In the early stage of T1DM, insulin is normally secreted by β cells, but the abnormal and increased demand for insulin in the β cells results in insulin failure (11,12). There are numerous therapies for T1DM and T2DM, but none are able to cure the disease. Moreover, the complications of diabetes can be serious, although there are therapeutic agents that can be used

to address these complications. For example, diabetic retinopathy is a complication that may result in a significant reduction in visual acuity (13), and results from neovascularization in the retina and cornea (14,15). In clinical studies (16-19), DM has been shown to reduce tear film quality and impair corneal sensation, and diabetic patients may experience discomfort, particularly dry eye disease (DED). According to a previous study, diabetic patients suffer autonomic dysfunction caused by chronic inflammation, which can manifest as DED (19).

DED is a common ocular disease characterized by itching, discomfort, pain and impaired tear production (20,21). DED has various causes, such as computer use, contact lenses, infection, age and environmental damage (22). The eyes of mammals comprise the cornea, iris, lens, retina, choroid, sclera, conjunctiva and ocular adnexa, including the lacrimal gland, lacrimal canaliculus, lacrimal sac and nasolacrimal duct (23). Increased angiogenesis is observed in the cornea and lacrimal gland of patients with DED, which is comparable with that in diabetic retinopathy (24,25). As DED progresses, the cornea becomes thinner and the ocular surface becomes more sensitive to the external environment (26). In this manner, ocular cells can be damaged and lose their function (27). The cells of the lacrimal gland that secrete the aqueous tear fluid may also be damaged (28). Current therapies for DED include oily eye drops, preservative-free drops, anti-inflammatory treatments and surgery (28-30).

Reactive oxygen species (ROS) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase contribute to diabetes complications (31-33), and NADPH oxidase (NOX) species also play important roles in the generation of ROS in diabetes (34,35). NOX species are enzymes that have the capacity to transport electrons across the plasma membrane and to generate superoxide and other downstream ROS (34). NOX species include NOX1-5 and dual oxidase 1/2 (36). ROS are upregulated due to sunlight, microbial antigens, pollutants, hormone and age, and are downregulated by various antioxidant proteins (37,38). However, if the signaling pathways associated with the antioxidant defense are disrupted, ROS levels increase and damage occurs to the tear lipid layer as well as epithelial and goblet cells in ocular surface, which can induce vascular endotheliopathy, corneal neuropathy and reduced tear secretion (39,40).

As oxidative stress upregulates ROS and NOX, antioxidant molecules such as carnosine and hyaluronic acid are potential treatments for the reduction of ROS and NOX levels (41,42). Previous studies of the pan-NOX inhibitor APX-115A have demonstrated its potential as a therapeutic agent for diabetes in mouse models. Based on this, the present study aimed to investigate the potential of APX-115A as a therapy for dry eye syndrome due to diabetes as an alternative to other models involving an impaired evaporative tear film (43-45). Moreover, it has been reported that the inhibition of ROS by treatment with APX-115A in Epstein Barr virus-infected ARPE-19 cells induces caspase-dependent apoptosis by activating the ERK-JNK pathway (46). In the present study, the effects of APX-115A on NOX2 expression were evaluated in a diabetic rat model (47). NOX2 is the phagocyte NADPH oxidase that is secreted by the cornea and lacrimal gland and is activated during phagocytosis to produce superoxide (48), while NOX4 is a phagocyte-type oxidase that modulates the ROS level in diabetes (49). Therefore, the present study investigated whether DED induced by diabetes can be relieved by the pan-NOX inhibitor, APX-115A, and evaluated the efficacy of APX-115A as a downregulator of ROS levels in a diabetic rat model (45).

Materials and methods

Reagents. Streptozotocin (STZ) and 0.1 M citrate buffer were obtained from Sigma-Aldrich (Merck KGaA) and BioPrince (Tech&Innovation), respectively. Primary antibodies against NOX2 were obtained as a gift from Professor Bae of Ewha Woman's University (Seoul, South Korea). APX-115A, a pan-NADPH oxidase inhibitor, was synthesized and provided by AptaBio Therapeutics Incorporation.

Animals and eye drop treatment. Male Sprague Dawley rats (6 weeks of age, >200 g) were purchased from Orient Bio Inc. The animal study was granted and approved by the Institutional Animal Care and Use Committee of Inje University College of Medicine (Busan, Korea; approval no. 2016-11), and all procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All rats were kept on a 12-h alternating light/dark cycle at 22±2˚C and 55-60% humidity. All animals had free access to sterile food and sterile tap water during this study. For the study, the rats were divided into four groups as follows: Normal saline (n=7), normal rats treated with normal saline; normal APX-115A (n=7), normal rats treated with APX-115A; diabetic saline (n=7), diabetic rats treated with normal saline; and diabetic APX-115A (n=7), diabetic rats treated with APX-115A. Diabetes was induced by the intraperitoneal injection of 60 mg/kg STZ in a citrate buffer (100 mM sodium citrate and 100 mM citric acid, pH 4.5) in a single dose after the rats had fasted for 6 h (50). The body weights and blood glucose levels of the rats were measured once a week for 6 weeks. APX-115A was dissolved in normal saline at a concentration of 10 mg/ml and 20 μl was applied to the rat eyeballs three times each day (at 9:00, 13:00 and 18:00) for 1 month.

Tear volume measurements. Reduced tear volume is one of the main characteristics of DE syndrome (21). To determine whether APX-115A prevents reduced tear volume in STZ-induced diabetic rats, tear volume was measured using a phenol red thread (PRT) tear test with Zone-Quick (Menicon Co., Ltd.) (51). The thread was placed into the palpebral conjunctiva for 15 sec, and then the entire length of the red portion of the thread was measured. Tear volume was measured once a week for 1 month. All rats were sacrificed humanely at 6 weeks after STZ injection using a CO_2 chamber with a CO_2 fill rate of 60% chamber volume/min (chamber volume, 15.73 liters; flow rate, 9.44 l/min). The lacrimal glands and eyeballs were collected and the lacrimal glands were weighed.

Hematoxylin and eosin (H&E) staining. The eyeballs and lacrimal gland tissues were fixed in 4% neutral formaldehyde solution at 4˚C overnight and formed into paraffin-embedded blocks using a Tissue Processor (TP 1020; Leica Microsystems, Inc.) according to a programming worksheet (alcohol and

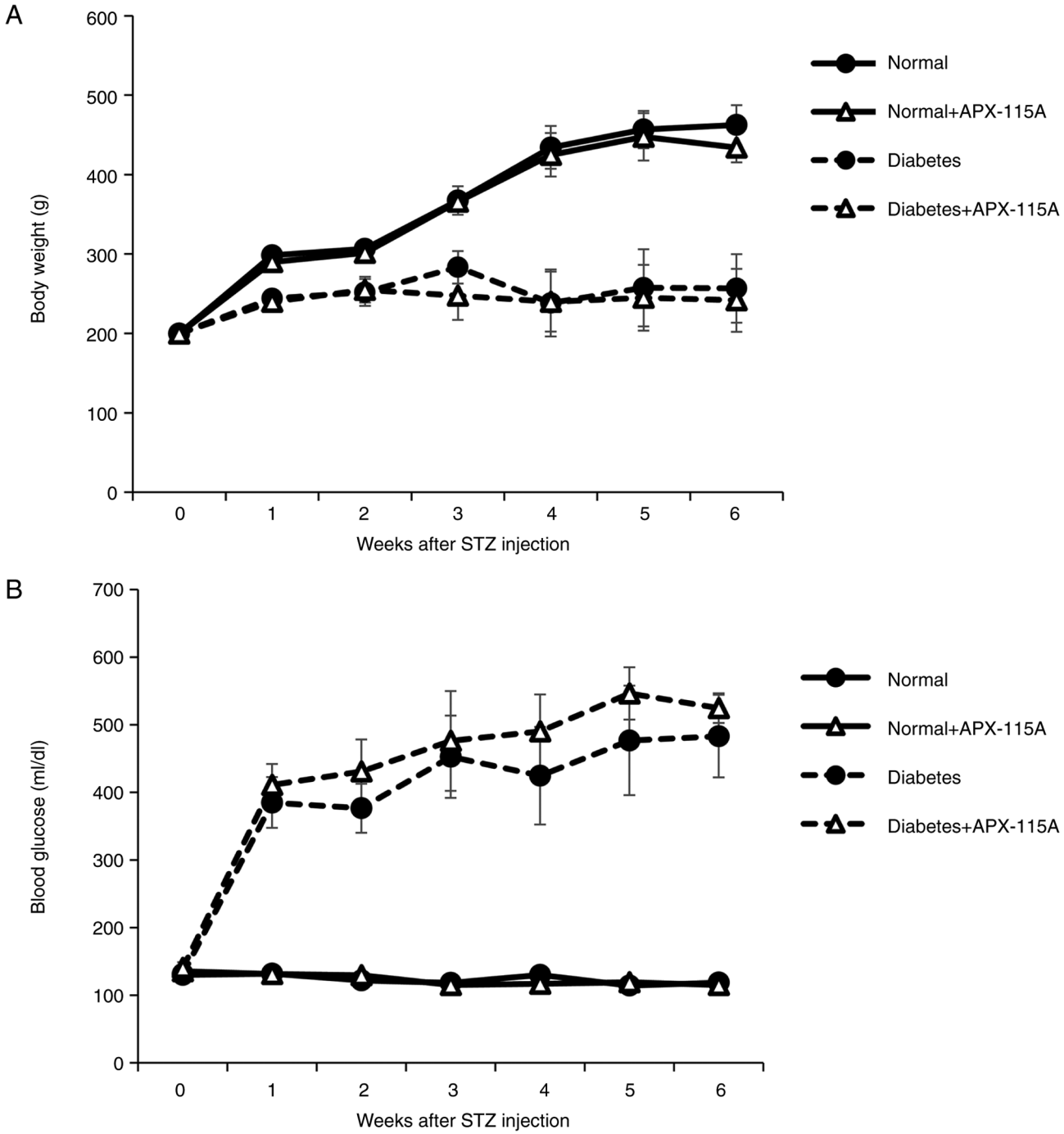

Figure 1. Blood glucose levels and body weights of the rats in the four study groups. (A) Body weights and (B) blood glucose concentrations were measured once weekly for 6 weeks after the induction of diabetes using STZ (n=6 per treatment group). STZ, streptozotocin.

xylene series; starting in 70% alcohol for 1 h and ending in xylene for 2 h) and embedding system (EG1150 model; embedding temperature, 65˚C). The blocks were sectioned into 7-μm cross sections and subjected to H&E staining using an Autostainer (Autostainer RSt model; Vision BioSystems, Inc.) according to the programming worksheet (briefly staining with alum hematoxylin for 20 min, followed by washing steps, and staining with 1% eosin Y for 5 min). The corneal thickness was measured using Image viewing software (Japan NDP. view2; Hamamatsu Photonics K.K.) after slide images were digitized with a Nanozoomer Virtual Microscope (Hamamatsu Photonics K.K.).

Immunohistochemistry. After deparaffinization (briefly, sections were treated with xylene for 2 h, and then rehydration processing was performed with a descending alcohol series at room temperature) and antigen blocking using antigen retrieval solution (cat. no. S2369; Dako; Agilent Technologies, Inc.) for 30 min at room temperature according to the manufacturer's instructions, the samples were incubated with anti-NOX2 (1:200 dilution) antibodies at 37˚C for 1.5 h. After washing three times with TBS, the samples were incubated with peroxidase-conjugated goat anti-mouse/rabbit IgG antibody (1:500 dilution; K5007 EnVision Detection System; Dako; Agilent Technologies, Inc.) at 37˚C for 1 h.

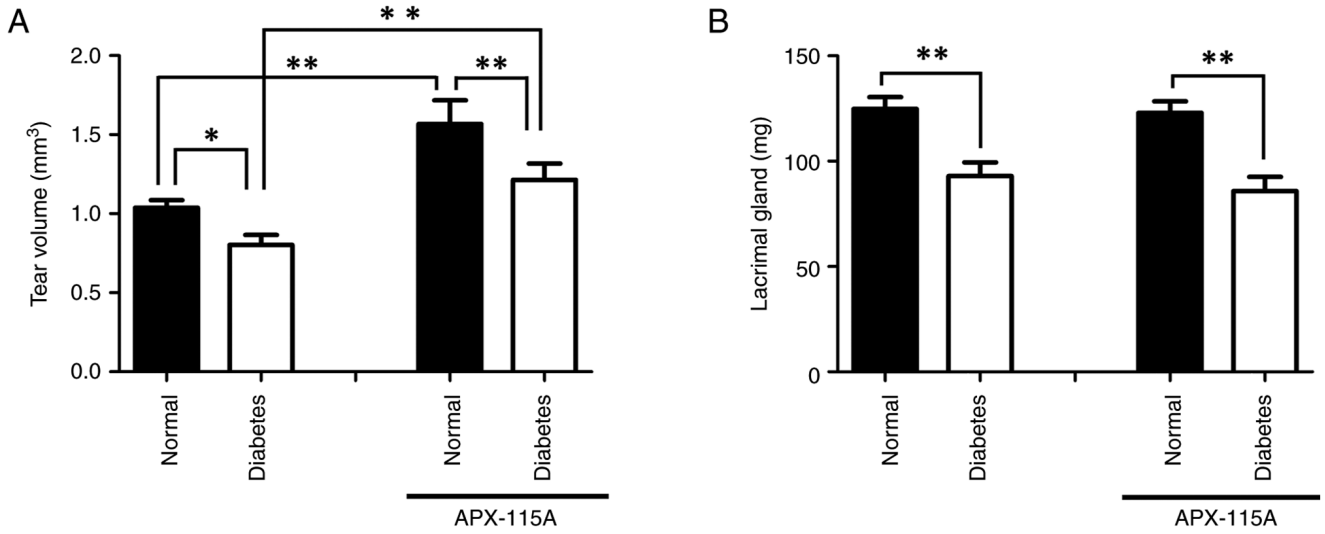

Figure 2. Tear volume and lacrimal gland weight. (A) Tear volume was reduced in the diabetic control group compared with the normal saline-treated group, but was increased in the diabetic rats following treatment with APX-115A. (B) Lacrimal gland weights were measured. Two-way ANOVA with Bonferroni post hoc test was performed, and statistically insignificant comparisons are not indicated. Lacrimal gland weights were reduced in both diabetic groups compared with the respective normal groups. *P<0.05, **P<0.01.

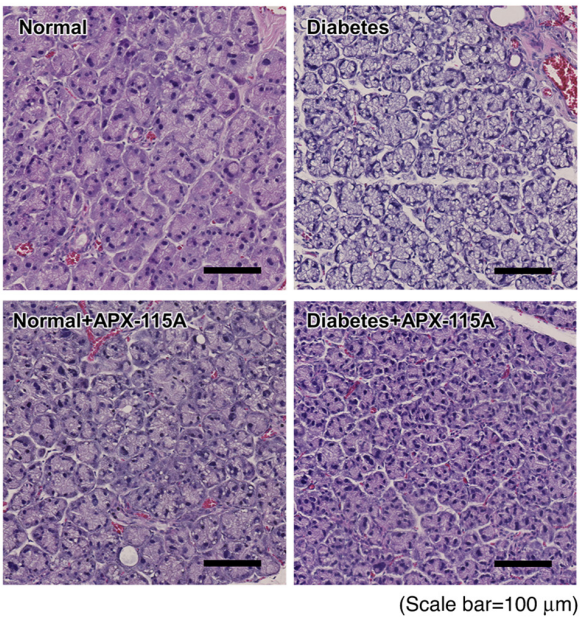

Figure 3. Effect of APX-115A on the morphology of the lacrimal glands in normal and diabetic rats. Representative photomicrographs of hematoxylin and eosin stained lacrimal glands show morphological changes. Numerous vacuoles and partial acinar atrophy were observed in the only lacrimal gland of the diabetes group, but APX-115A treatment restored these morphological changes (magnification, x400; scale bar, 100 μm).

DAB was used to visualize the target NOX2, while Meyer's hematoxylin was used for 5 min at room temperature (Dako; Agilent Technologies, Inc.) to visualize the nucleus. The images were digitized with a Hamamatsu Nanozoomer Virtual Microscope (Hamamatsu Photonics K.K.) and visualized at high resolution with the aid of NDP View2 software (Hamamatsu Photonics K.K.).

Statistical analysis. DAB-positive areas (NOX2-positive areas) were measured using ImageJ 1.53 software (National Institutes of Health) and percentages of area are expressed as the mean ± standard error of the mean. For all comparisons among multiple groups, data were analyzed using a two-way ANOVA with Bonferroni post hoc tests. P<0.05 was considered to indicate a statistically significant difference.

Results

APX-115A has no effect on body weight or blood glucose level. To determine whether APX-115A affected body weight and blood glucose in an STZ-induced diabetic model, rats were induced to develop DM using STZ in a citrate buffer by intraperitoneal injection (50). After the induction of DM, APX-115A was applied to the rat eyes in drop form three times a day for 1 month. The body weights and blood glucose levels of the rats were measured once a week for 1 month (Fig. 1). The results showed reduced body weights (Fig. 1A) and increased blood glucose levels (Fig. 1B) in the diabetic rats compared with the normal rats. No differences in body weight or blood glucose level were observed between the diabetic controls and the diabetic rats treated with APX-115A. These results demonstrate that APX-115A treatment had no effect on the body weights and blood glucose levels of the rats.

APX-115A prevents reduced tear volume in STZ-induced diabetic rat models. Reduced tear volume is one of the main characteristics of DE syndrome (21). To determine whether APX-115A affects the reduced tear volume in STZ-induced diabetic rats, tear volume was measured using a PRT tear test. Tear volume was reduced in the diabetic control group compared with the normal control group; however, there was a significant increase in tear volume in the normal and diabetic rats treated with APX-115A compared with the respective saline-treated controls (Fig. 2A). The weights of the lacrimal glands were decreased in both diabetic groups compared with the respective saline-treated controls, and APX-115A treatment

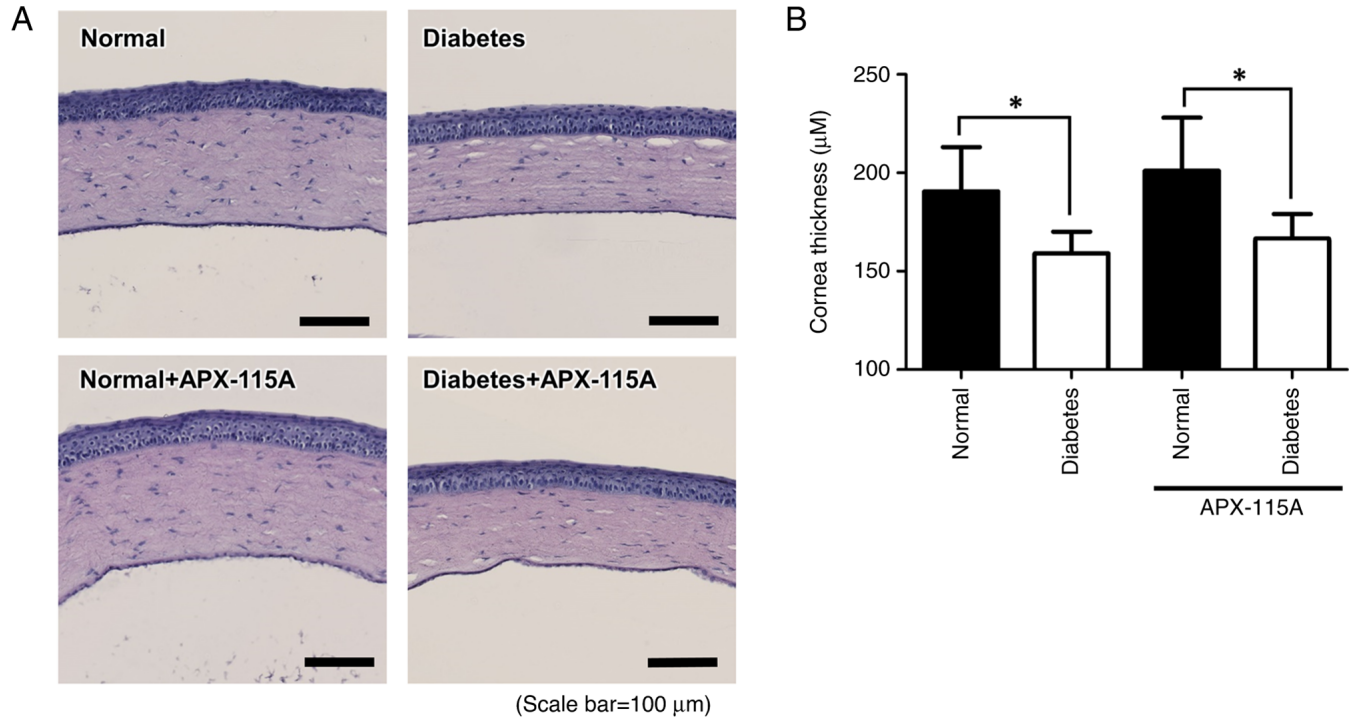

Figure 4. Effect of APX-115A on corneal thickness in normal and diabetic rats. (A) Representative images of hematoxylin and eosin stained eyeball tissue showing the thickness of the cornea in the normal control, normal + APX-115A, diabetic control and diabetic + APX-115A groups (x400 magnification; scale bar, 100 μm). (B) Corneal thickness was measured (n=20/group). Two-way ANOVA with Bonferroni post hoc test was performed, and statistically insignificant comparisons are not indicated. The thickness of the cornea in both diabetic groups was reduced compared with that in the corresponding normal group. *P<0.05.

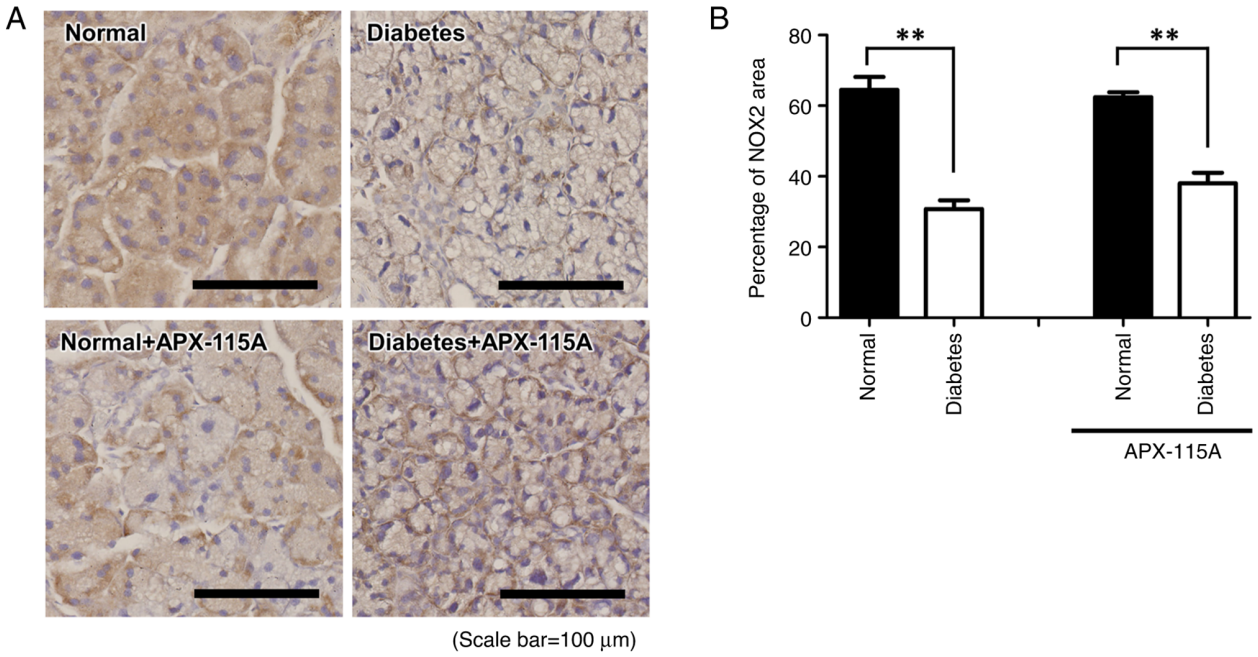

Figure 5. Evaluation of NOX2 expression in the lacrimal glands of normal and diabetic rats with and without APX-115A treatment, as assessed using immunohistochemistry (A) Representative images showing the immunochemistry of NOX2 in the lacrimal glands (magnification, x400, scale bar, 100 μm). (B) NOX2 positivity was measured as a percentage of the total area. Two-way ANOVA with Bonferroni post hoc test was performed, and statistically insignificant comparisons are not indicated. NOX2 expression in the lacrimal gland was reduced in both diabetic groups compared with the corresponding normal group. **P<0.01. NOX2, nicotinamide adenine dinucleotide phosphate oxidase 2.

exhibited no significant effect (Fig. 2B). These results indicate that APX-115A administration increased the tear volume of the rats but had no effect on lacrimal gland size.

APX-115A restores morphological changes in lacrimal glands induced by DM. To investigate how ocular mucosal inflammation is affected by DED (52,53), the morphologies of the

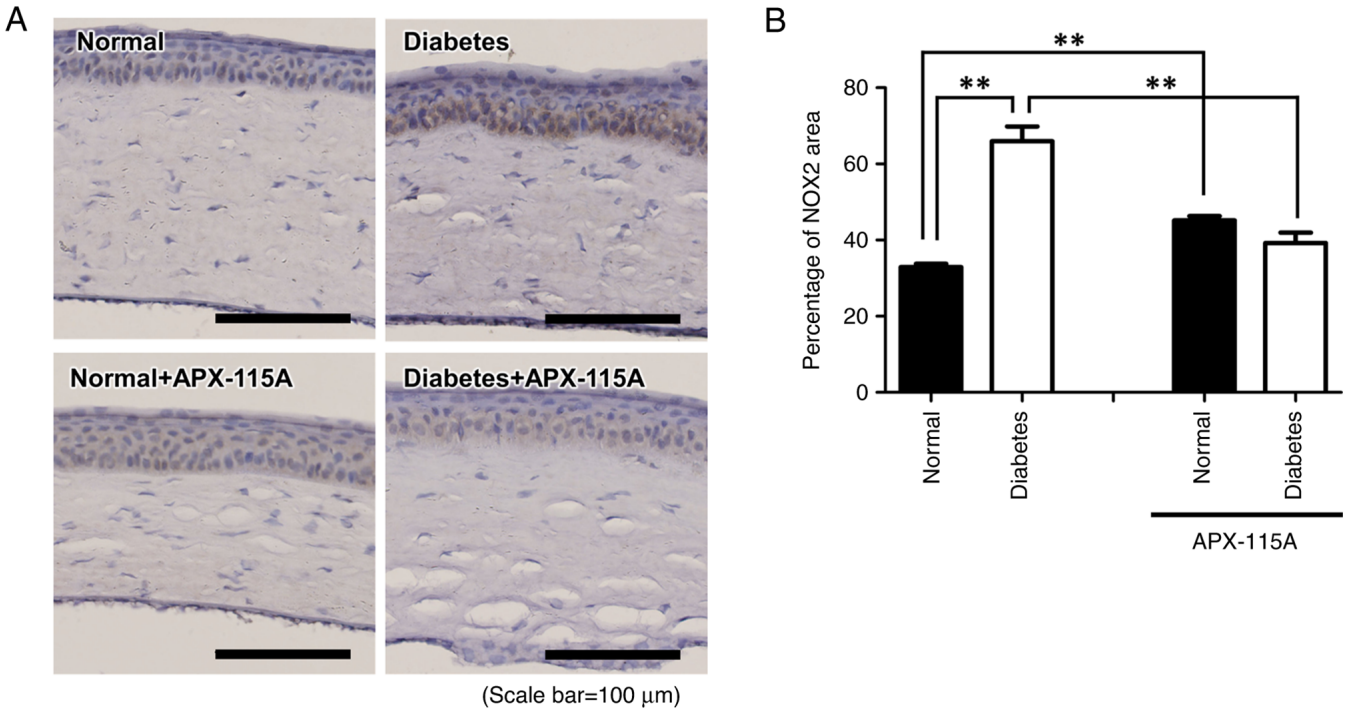

Figure 6. Evaluation of NOX2 expression in the corneal epithelial layers of normal and diabetic rats with and without APX-115A treatment. (A) Representative images showing the immunochemistry of NOX2 in the cornea (magnification, x400, scale bar, 100 μm). (B) NOX2 positivity was measured as a percentage of the area of the corneal epithelial layer. Two-way ANOVA with Bonferroni post hoc test was performed, and statistically insignificant comparisons are not indicated. NOX2 expression in the corneal epithelial layer was increased in the diabetic group compared with the normal group in the rats that did not receive APX-115A treatment, but NOX2 expression was reduced in the diabetic rats following APX-115A treatment. **P<0.01. NOX2, nicotinamide adenine dinucleotide phosphate oxidase 2.

lacrimal glands were examined using H&E staining. The lacrimal glands of the diabetic controls contained numerous vacuoles and exhibited partial acinar atrophy, but these morphological changes were ameliorated in the diabetic rats treated with APX-115A (Fig. 3). These results indicate that APX-115A inhibited the lacrimal gland from forming vacuoles and undergoing acinar atrophy.

APX-115A does not recover corneal thickness. Corneal thickness is significantly decreased in DED (54). To determine whether treatment with APX-115A affected the corneal thickness in STZ-induced diabetic rats, sectioned eyeball tissues were examined by H&E staining. The corneal stromal layers in the STZ-induced diabetic rats were decreased compared with those in the respective normal rats. APX-115A treatment did not attenuate the reduction in corneal thickness (Fig. 4). These results revealed that the corneal thickness of the eyeballs from rats with DM-associated DED was reduced, and APX-115A had no effect on corneal thickness.

APX-115A reduces corneal NOX2 levels. To evaluate whether APX-115A reduced NADPH oxidase levels and thereby ameliorated ocular inflammation, sectioned lacrimal gland and eyeball tissues were subjected to NOX2 immunochemistry (Figs. 5 and 6). In the lacrimal gland, the level of NOX2 was significantly lower in the diabetic controls compared with the normal controls, and no significant difference was detected between the diabetic controls and the diabetic rats treated with APX-115A (Fig. 5). In contrast with the results for the lacrimal gland, the level of NOX2 was increased in the epithelial layer of the cornea in diabetic rats compared with the normal controls, and APX-115A treatment significantly attenuated the diabetes-induced increase in NOX2 level (Fig. 6).

Discussion

DM is a common disease and has an increasing incidence (2). This disease is associated with numerous complications, including diabetic retinopathy, diabetic kidney disease and diabetic neuropathy. Treatments for these complications are limited, which has prompted research aiming to address this deficiency. DED syndrome is a complication of DM, which is associated with dysfunction of the corneal layers and lacrimal glands. Due to various causes (mainly dysfunction of meibomian glands, aging, ocular and general diseases, including DM, contact lens wear and adverse environment exposure), the lipid layer and aqueous layer of the tear film lose thickness, and the epithelial layer forms new blood vessels (55-57).

APX-115 is a pan-NOX inhibitor that has been investigated as a treatment for diabetic complications (45,58). The present study investigated the efficacy of APX-115A as a treatment for DED in diabetic rats. The STZ-induced diabetic rat is a good model for diabetes because it demonstrates various symptoms in a short period of time, including hyperglycemia and reduced body weight. Moreover, the STZ-induced diabetic rat model has been used in numerous studies such as those investigating diabetic retinopathy and DM pathogeneses, including endoplasmic reticulum stress and oxidative stress (59-61). APX-115A had no effects on blood glucose level and body weight in the present study, which is in accordance with a previous study (58).

However, APX-115A increased the tear volume compared with that in the saline-treated controls. Previous studies have shown that ROS inhibitors such as APX-115A can restore tear secretion to a normal level (45,62-64). Furthermore, pathological alterations were detected in the cornea and lacrimal glands of the diabetic rat models. The relationship between changes in cornea thickness and diabetic dry eye remains unclear, but studies have demonstrated that reduced corneal epithelial thickness is a diabetic complication (65,66). However, in the present study, the change in corneal thickness in diabetic rats was not reversed, although the intracellular vacuoles and acinar atrophy were ameliorated by APX-115A treatment in the diabetic rats. These observations indicate that APX-115A can prevent DED at an early stage.

Oxidative stress, such as that produced by NOX and ROS, plays a vital role in diabetic complications. NOX2 is an NADPH oxidase isoform (47). Increased expression of NOX2 has been observed in cells with a high ROS level (46). In the present study, NOX2 levels in sectioned corneal and lacrimal gland tissues were evaluated using immunohistochemistry. Positive effects of APX-115A on NOX2 were noted, as the level of NOX2 in the corneal epithelium of APX-115A-treated diabetic rats was decreased compared with that in the saline-treated diabetic group. The expression of NOX2 in the lacrimal gland was particularly reduced in the proximity of blood vessels. Although the corneas were directly treated with APX-115A using an eye drop method, it is challenging to apply APX-115A directly to the lacrimal gland. This may explain why the NOX2 expression level in the cornea was strongly regulated while that in the lacrimal gland was weakly regulated in diabetic rats treated with APX-115A. Moreover, it has previously been shown that meibomian glands, which are important for maintenance of the tear film and are associated with the condition and integrity of the ocular surface (67), are influenced by APX-115A eye drops, which protect the tear film structure and attenuate DED (68). The conjunctiva is an important physical barrier for protecting against ocular inflammation (69). Morphological changes of the conjunctiva have been demonstrated due to dry eye in diabetic rats (70), but APX-115A has little effect on the conjunctiva. Further studies are required to evaluate the effects of APX-115A on conjunctival inflammation in diabetic dry eye.

In summary, the present study showed that APX-115A protects against the early stages of DED due to diabetes by inhibiting NOX2 expression in the cornea and attenuating morphological changes of the lacrimal glands.

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Availability of data and materials

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Authors' contributions

SYH, SHM and DYH were responsible for conceptualization, and for writing, reviewing and editing the manuscript. MHN and DKL were responsible for data collection. MHN and YSK performed the formal analysis. SYH acquired funding. MHN performed the experiments. SHM and DYH were responsible for study design. MHN wrote the original draft of the manuscript. HYK performed statistical analysis and revised the manuscript. MHN and DYH confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The animal experiments were granted and approved by the Institutional Animal Care and Use Committee of Inje University College of Medicine (approval number: 2016-11), and all procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Patient consent for publication

Not applicable.

Competing interests

SHM is an employee of Aptabio Therapeutics Inc., Republic of Korea, which is developing APX-115A. The remaining authors declare that they have no competing interests.

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