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Research Paper

Protection against advanced glycation end products and oxidative stress during the development of diabetic keratopathy by KIOM-79

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Abstract

Objectives KIOM-79 is a mixture of 80% ethanol extracts of parched Puerariae radix, gingered Magnoliae cortex, Glycyrrhizae radix and Euphorbiae radix. The preventive effect of KIOM-79 on the development of diabetic keratopathy has been investigated.

Methods Seven-week-old male Zucker diabetic fatty (ZDF) rats were treated with KIOM-79 (50 mg/kg body weight) once a day orally for 13 weeks. The thickness of the cornea was measured and the extent of corneal cell death was detected by a terminal deoxynucleotidyl transferase dUTP nick-end labelling assay. The expression of advanced glycation end products (AGEs), 8-hydroxydeoxyguanosine, nuclear factor-kappaB (NF-κB), Bax and Bcl-2 were evaluated in corneal tissues.

Key findings The administration of KIOM-79 prevented corneal oedema and apoptotic cell death of corneal cells. The accumulation of AGE in corneal tissues was reduced in ZDF rats treated with KIOM-79. Moreover, KIOM-79 attenuated oxidative DNA damage, NF-κB activation and Bax overexpression in the cornea.

Conclusions The results suggested that KIOM-79 exhibited corneal protective properties by not only reducing oxidative stress but inhibiting the AGEs/NF-κB downstream signal pathway during the development of diabetic keratopathy.

Keywords advanced glycation end products; apoptosis; diabetic keratopathy; KIOM-79

Introduction

Hyperglycaemia is the major cause of diabetic complications, such as retinopathy, nephropathy and neuropathy.[1–3] Diabetic keratopathy is one of the most common ocular complications of diabetes. Recently, advanced glycation end products (AGEs), the result of sugar-derived irreversible protein modifications, have been implicated as one of the mechanisms that create diabetic corneal complications.[4,5] It has been shown that in patients with diabetes, AGEs can increase abnormally and accumulate on tissue and organs, causing chronic complications for the patient.[6] In the human eye, AGEs were detected in the corneal stroma, lens, Descemet's membrane, basement membrane of the corneal epithelium and the lamina cribrosa.[4,7–9] Toxic effects of AGEs result from structural and functional alterations in the plasma and the extracellular matrix proteins, particularly from the cross-linking of proteins and the interaction of AGEs with their receptors (RAGE). It has been reported that the interaction between AGEs and their receptors causes enhanced formation of oxygen radicals, which subsequently activate nuclear factor-κB (NF-κB) and cause the release of pro-inflammatory cytokines.[10–13] Based on these results, it is likely that the inhibitors of AGEs could be potential therapeutics in the prevention of diabetic keratopathy.

KIOM-79 consists four medicinal herbs, Puerariae radix, gingered Magnoliae cortex, Glycerrhizae rhizoma and Euphorbiae radix. This herbal mixture was developed because each herb has been used historically in Korea and other countries in the treatment of diabetes or diabetic complications.[14–17] In our previous studies, KIOM-79 showed a strong inhibitory effect on AGE formation, reduced AGE accumulation in the kidney and reduced the development of diabetic nephropathy in an animal model for type 1 and type 2 diabetes.[18,19] In addition, KIOM-79 demonstrated anti-inflammatory properties by blocking NF-κB and p38 kinase activation in a murine macrophage cell.[20] KIOM-79 also demonstrated cytoprotec-

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tive properties by acting as an antioxidant via haem oxygenase-1 induction in a pancreas-beta cell line.[21] These various pharmacological effects of KIOM-79 may be useful for the treatment or prevention of diabetic keratopathy. Herein, we have investigated the effects of KIOM-79 on diabetic corneal complications in a Zucker diabetic fatty (ZDF) rat, a rat model for type 2 diabetes.

Materials and Methods

Preparation of KIOM-79

The cortex of *Magnolia officinalis*, radix of *Pueraria lobata*, radix of *Glycyrrhiza uralensis* and radix of *Euphorbia pekinensis* were collected from plants obtained from the Gamsuk Province (China) and identified by Professor J. H. Kim (Division of Life Science, Kyungwon University, Korea). KIOM-79 was prepared as reported by Jeon *et al*.[20] All voucher specimens were deposited at the herbarium of the Korea Institute of Oriental Medicine (No. 1240, 2, 7 and 207, respectively).

Animal experimental design

Male six-week-old ZDF rats (ZDF/Gmi-fa/fa) and Zucker lean (ZL) counterparts (ZDF/Gmi-lean) were purchased from Charles River Laboratory (Waltham, MA, USA). Rats received a diet of Purina 5008 (Ralston Purina, St Louis, MO, USA) and tap water was freely available. At seven-weeks of age, they were divided into the following three groups of eight rats: ZL rats; untreated ZDF rats; and ZDF rats treated with KIOM-79 (50 mg/kg body weight). KIOM-79 was dissolved in water and given orally. To the untreated ZDF rats and ZL rats, saline was given orally for 13 weeks. The blood glucose level was monitored periodically. All procedures involving rats were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the Korea Institute of Oriental Medicine Institutional Animal Care and Use Committee (IACUC approval no. 2009-013).

Histopathological analysis

Thirteen weeks after oral administration, the eyes were enucleated from the animals, fixed in 10% phosphate-buffered formalin for 24 h and embedded in paraffin wax. The eyes were then cut in 4-μm thick sections and stained with haematoxylin and eosin (H&E). To analyse the corneal oedema, corneal tissues were visualized by labelling with rhodamine-conjugated wheat germ agglutinin (Vector Laboratories, CA, USA). The thicknesses of the total cornea, corneal epithelium and stroma were measured in the central region of each cornea.

Apoptosis assay

The TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labelling) assay was performed using a commercially available kit (DeadEnd apoptosis detection system, Promega, Madison, WI, USA) according to the manufacturer's instructions. Apoptotic cells were detected using fluorescein-conjugated streptavidin. For quantitative analysis, TUNEL-positive nuclei were then counted per unit area (0.32 mm^2) in a total of five fields.

Corneal autofluorescence measurement

Corneal autofluorescence was measured as previously described.[5] Briefly, corneas were homogenized in phosphate-buffered saline and autofluorescence was determined at an excitation wavelength of 340 nm and an emission wavelength of 460 nm using a spectrofluorometer (Synergy HT, BioTek, Woburn, MA, USA). Fluorescence intensity was expressed per milligram of protein. Protein concentrations in all samples were measured spectrophotometrically using a Lowry assay with bovine serum albumin as standard.

Immunohistochemical staining

Immunohistochemistry was performed as previously described.[22] Briefly, antibodies were mouse anti-AGEs (Cosmo bio, Tokyo, Japan) and rabbit anti-8-hydroxydeoxyguanosine (8-OHdG, Abcam, MA, USA). For detection of AGEs and 8-OHdG, the sections were incubated with the LSAB kit (DAKO, CA, USA) and visualized using 3,3′-diaminobenzidine tetrahydrochloride. The sections were counterstained with haematoxylin. The stained sections were visualized on a computer display with an Olympus DP71 camera connected to an Olympus light microscope (Tokyo, Japan).

Southwestern histochemistry for detection of activated NF-κB

Complementary oligonucleotides containing an NF-κB binding consensus sequence were synthesized as follows: 5′-AGTTGAGGGGACTTTCCCAGGC-3′. The probe was labelled with digoxigenin (DIG oligonucleotide 3′-end labelling kit, Roche Diagnostics, Mannheim, Germany). The eye sections were rehydrated and subsequently digested with pepsin A (433 U/mg; Sigma). The labelled probe (100 pM) was applied overnight at 37°C. After washing, sections were incubated with antidigoxigenin antibody conjugated with alkaline phosphatase (Roche Diagnostics, Mannheim, Germany). The sections were then incubated in the dark for 3–4 h with a colour solution containing nitroblue tetrazolium (NBT, Roche Diagnostics) and 5-bromo-4-chloro-3-indolylphosphate (BCIP, Roche Diagnostics). As negative controls, the following conditions were used: the absence of probe; a mutant NF-κB probe labelled with digoxigenin; and competition assays with a 200-fold excess of unlabelled NF-κB followed by incubation with labelled probe.

Evaluation of NF-κB DNA binding activity

Nuclear proteins were isolated from the cornea using nuclear and cytoplasmic extraction kits (NE-PER Nuclear and Cytoplasmic Extraction Reagents, Pierce, IL, USA). NF-κB DNA-binding activity was evaluated using an ELISA-based EZ-detected Transcription Factor Kit for NF-κB p65 (Pierce) according to the manufacturer's instructions. All samples were run in triplicate.

Western blot analysis

Proteins were extracted from corneas, separated using SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Biorad, CA, USA). Membranes were probed with anti-Bax antibody (Santa Cruz, CA, USA) and

anti-Bcl-2 antibody (Santa Cruz). The immune complexes were then visualized with an enhanced chemiluminescence detection system (ECL, Amersham Bioscience, NJ, USA). Protein expression levels were determined by analysing the signals captured on the nitrocellulose membranes using an image analyser (Las-3000, Fuji Photo, Tokyo, Japan).

Statistical analysis

Statistical evaluation of the results was performed using a two-tailed Student's *t*-test and a one-way analysis of variance followed by Tukey's multiple comparison test using GraphPad Prism 4.0 software (GraphPad, CA, USA).

Results

Fasting blood glucose level

At 21 weeks of age, all ZDF rats developed hyperglycaemia compared with the normal ZL rats. As shown in Table 1, the untreated ZDF rats had more than a fourfold increase in their fasting blood glucose level. KIOM-79 induced a minor decrease in blood glucose levels.

Histopathological analysis

In the cornea of the untreated ZDF rats, perinuclear clear areas were observed and the stroma was oedematous. The basement membrane was thickened. The thicknesses of the total cornea of untreated ZDF rats were significantly higher than those of normal ZL rats. The thicknesses of the stroma had significantly increased in untreated ZDF rats. However, this histological change of the cornea in ZDF rats was prevented by treatment with KIOM-79 (Figure 1).

Apoptosis of corneal cells

In the cornea of normal ZL rats, TUNEL-positive cells were barely detected. In untreated ZDF rats, many TUNEL-positive cells and numerous fragmented nuclei were observed in the corneal epithelial, stromal and endothelial cells (Figure 2a and b). This result indicated that several corneal cells underwent apoptosis under diabetic conditions. However, the treatment with KIOM-79 prevented an increase in positive cells similar to that seen in normal ZL rats.

AGE accumulation in the diabetic cornea

The corneal autofluorescence values showed significant correlation with AGE levels in the corneal tissue from diabetic patients.[23] Thus, we measured the autofluorescence of the corneal AGEs. The autofluorescence level in untreated ZDF rats (9.93 ± 3.47) was significantly greater than that in normal ZL rats (4.08 ± 1.81), whereas KIOM-79 markedly decreased its level (6.25 ± 2.06) compared with untreated ZDF rats (Figure 2d). In addition, we examined the accumulation of AGEs in the cornea with immunohistochemistry. As

Table 1 Fasting blood glucose level in rats treated with or without KIOM-79

	ZL	ZDF	KIOM-79
Blood glucose (mmol/l)	5.16 ± 0.56	27.21 ± 2.11*	21.76 ± 6.29**

KIOM-79, Zucker diabetic fatty rats treated with KIOM-79 (50 mg/kg); ZDF, vehicle-treated Zucker diabetic fatty rats; ZL, normal Zucker lean rats. All data were expressed as the mean ± SD. *$P < 0.01$ compared with normal ZL rats, **$P < 0.05$ compared with untreated ZDF rats.

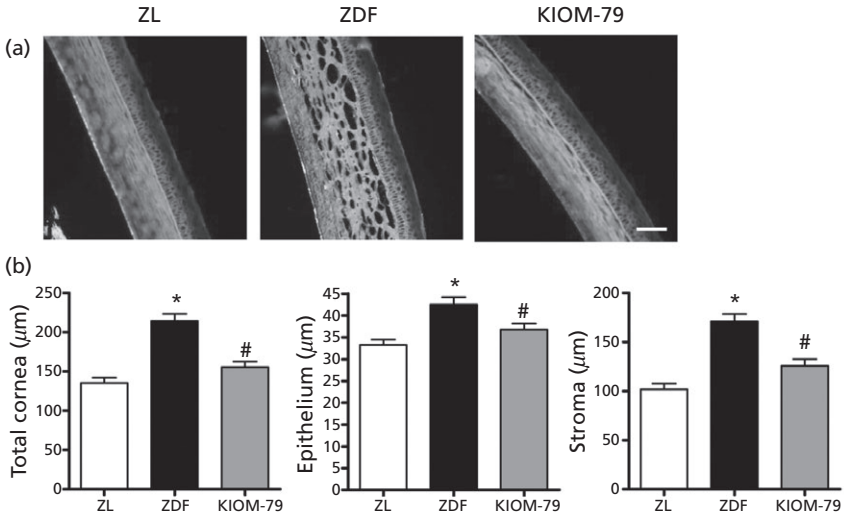

Figure 1 Analysis of corneal alteration in normal Zucker lean rats, and Zucker diabetic fatty rats treated with or without KIOM-79. (a) The corneal sections from the normal Zucker lean rat (ZL), untreated Zucker diabetic fatty rat (ZDF) and KIOM-79 treated ZDF rat (KIOM-79) were labelled with rhodamine-conjugated wheat germ agglutinin. The oedema of the corneal stroma was observed in diabetic corneas. Scale bar = 50 μm. (b) The thicknesses of the total cornea, epithelium and stroma were assessed. Values in the bar graphs represent means ± SE, $n = 8$. *$P < 0.01$ compared with normal ZL rats, #$P < 0.01$ compared with untreated ZDF rats.

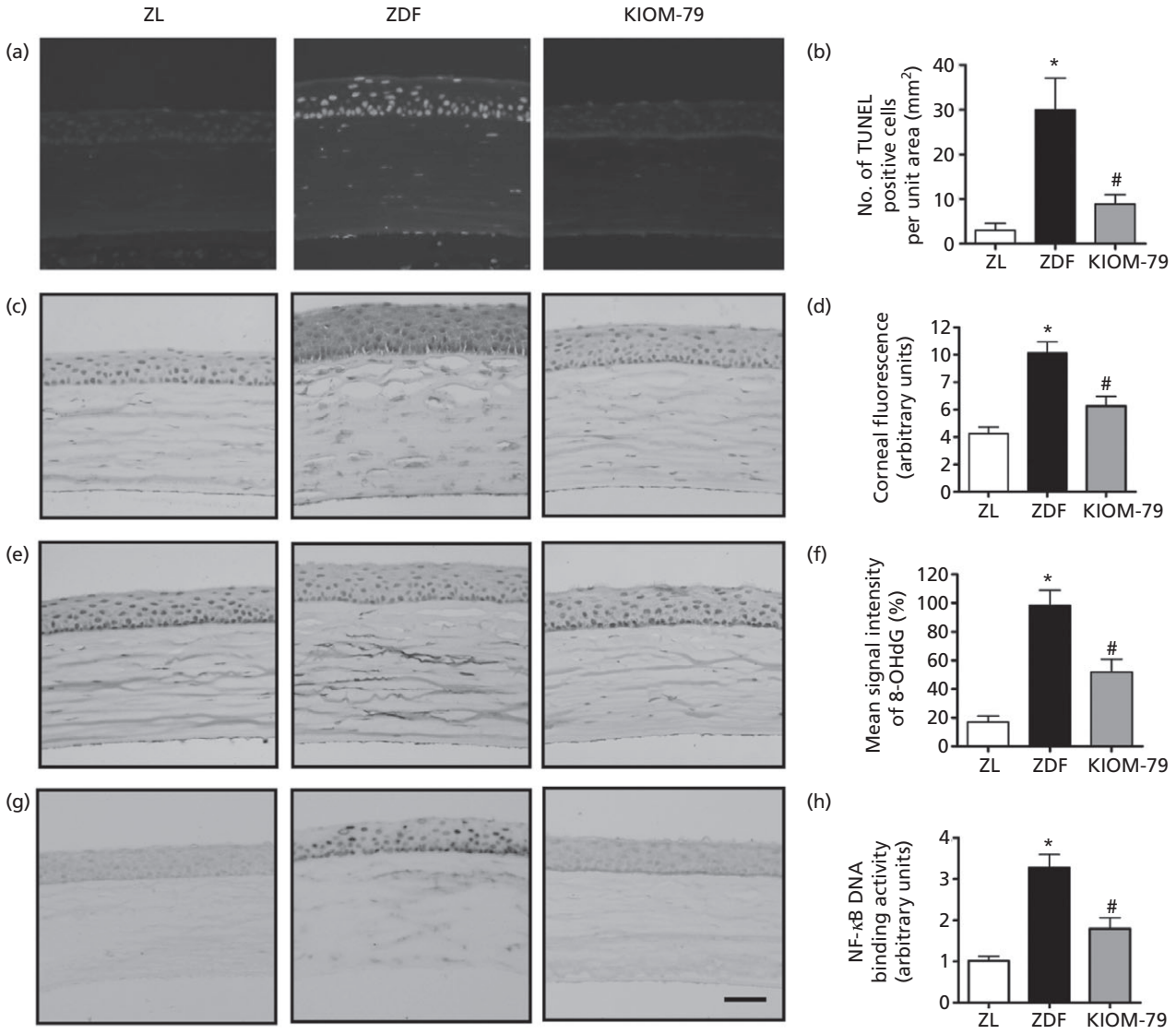

Figure 2 The effect of KIOM-79 on corneal alteration in normal Zucker lean rats, and Zucker diabetic fatty rats treated with or without KIOM-79. (a) TUNEL staining (green). (b) Quantitative analysis of TUNEL-positive cells. (c) Immunohistochemical localization of advanced glycation end products (AGEs). (d) AGEs autofluorescence values. (e) Immunostaining of 8-hydroxydeoxyguanosine (8-OHdG). (f) Quantitative analysis of 8-OHdG signal intensity. (g) Southwestern histochemistry of nuclear factor-kappaB (NF-κB). (h) NF-κB DNA binding activity by ELISA-based assay. Representative photomicrographs of corneas from the normal ZL rat (ZL), untreated ZDF rat (ZDF) and KIOM-79-treated ZDF rat (KIOM-79). Scale bar = 50 μm. All data are expressed as the mean ± SE, $n = 8$. *$P < 0.01$ compared with normal ZL rats, #$P < 0.01$ compared with untreated ZDF rats.

shown in Figure 2c, a striking increase in the immunoreactivity of AGEs was observed in the corneal epithelium, stroma and endothelium of untreated ZDF rats. However, the administration of KIOM-79 reduced the amount of AGEs present in the cornea.

Oxidative DNA damage in the diabetic cornea

To confirm the oxidative stress status in corneal cells under diabetic conditions, we assessed the formation of 8-OHdG, a marker indicative of oxidative stress. In untreated ZDF rats, an increased immunoreactivity of 8-OHdG showed nuclear and/or perinuclear localization in epithelial, stromal and endothelial cells. This expression of 8-OHdG followed a pattern similar to that of AGE accumulation. However, a remarkable reduction of 8-OHdG was observed in the cornea of KIOM-79-treated ZDF rats (Figure 2e and 2f).

Activation of NF-κB in the diabetic cornea

NF-κB activity was detected using Southwestern histochemistry. Normal corneas generally lacked activated NF-κB staining in any of the corneal layers, whereas in the cornea of untreated ZDF rats, marked NF-κB activity was found mainly in the corneal epithelial and endothelial cells. However, it was detected only weakly in stromal cells (Figure 2g). To confirm the results obtained from Southwestern histochemistry and to evaluate NF-κB activation quantitatively, we performed an ELISA-based NF-κB assay. Diabetic corneas exhibited a significantly higher activity of NF-κB than normal controls,

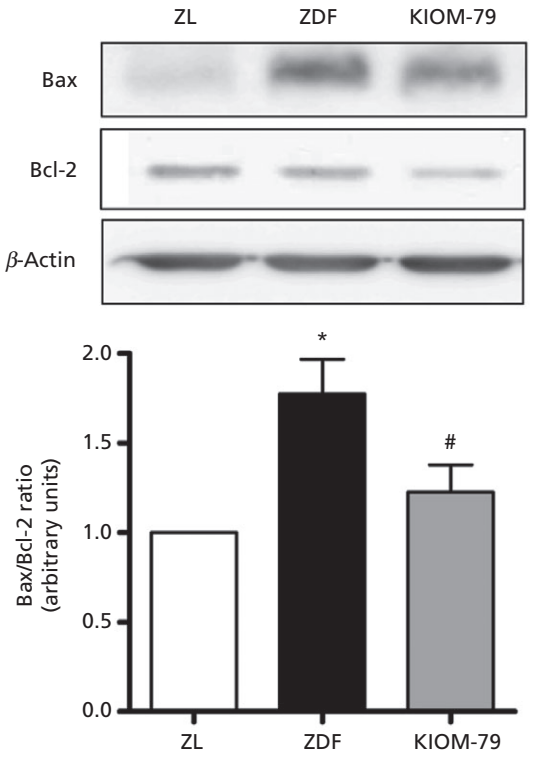

Figure 3 Expression of Bax and Bcl-2 proteins by Western blot and the ratio of Bax to Bcl-2 expression levels in rats treated with and without KIOM-79. Values in the bar graphs represent means ± SE, $n = 8$. *$P < 0.01$ compared with normal Zucker lean (ZL) rats, #$P < 0.01$ compared with untreated Zucker diabetic fatty (ZDF) rats. KIOM-79, ZDF rats treated with KIOM-79 (50 mg/kg).

whereas the level of activated NF-κB in KIOM-79-treated ZDF rats was significantly lower, by 60%, than those of untreated ZDF rats (Figure 2h).

Expression of Bax and Bcl-2 in diabetic cornea

To investigate the potent anti-apoptotic role of KIOM-79 further, we investigated the expression of pro-apoptotic Bax protein and anti-apoptotic Bcl-2 protein. Using Western blot analysis, the expression of Bax was significantly increased in the untreated ZDF rats compared with the normal ZL rats. The ratio of Bax to Bcl-2 in the ZDF rats was significantly higher than that in the normal ZL rats. However, treatment with KIOM-79 reduced the expression of Bax protein in the diabetic cornea (Figure 3).

Discussion

Ocular complications associated with diabetes include diabetic keratopathy.[24–26] It was reported recently that increased AGE accumulation contributed to the development of diabetic ocular complications, such as diabetic retinopathy and lacrimal gland dysfunction.[10,27–29] In this study, we have demonstrated the alteration of diabetic cornea, including increased corneal thickness and apoptosis of corneal cells. These changes were consistent with those described in similar animal studies and in humans with diabetes.[25,30,31] Enhanced AGE accumulation and oxidative DNA damage were also observed in the diabetic cornea. However, KIOM-79 prevented the damage of the cornea by inhibiting AGE accumulation and oxidative DNA damage in the corneas of ZDF rats. In our previous study, the administration of KIOM-79 to streptozotocin-induced diabetic rats (250 and 500 mg/kg) prevented the development of diabetic nephropathy in a dose-dependent fashion.[19] The administration of KIOM-79 (500 mg/kg) retarded the development of diabetic nephropathy in Goto-Kakizaki rats.[18] Diabetic db/db mice treated with KIOM-79 (150 mg/kg) showed a reduction in apoptotic cell death and lower AGE accumulation in retinas.[32] Recently, it was reported that long-term treatment with KIOM-79 (50 mg/kg) in the ZDF rat prevented the progress of diabetic nephropathy.[33] Based on those previous animal studies, we assumed that the minimum effective dose of KIOM-79 was 50 mg/kg body weight in diabetic animal models. Thus, in this study, we investigated the effect of KIOM-79 at a minimum dose of 50 mg/kg on diabetic keratopathy. Metabolic parameters, including blood glucose, low-density lipoproteins, high-density lipoproteins, triglyceride, and total cholesterol levels were significantly increased in ZDF rats, as previously described.[34] However, no difference in blood glucose or lipid levels were noted between KIOM-79-treated ZDF rats and untreated ZDF rats (data not shown).

The normal cornea maintains a constant thickness despite the fact that the corneal stroma has an inherent tendency to imbibe water. The corneal endothelium plays a key role in the prevention of stromal swelling.[35] Tight apical junctions in endothelial cells work as physical barriers, and ion pumps in the endothelium increase the movement of water outward from the corneal stroma to the anterior chamber.[35–37] Thus, corneal oedema can be induced as a result of a breakdown of either the anatomical barrier or the pump functions of corneal endothelial cells.

AGEs are a heterogeneous group of products that result from the nonenzymatic glycation and oxidation of proteins and lipids. The accumulation of AGEs in various tissues leads to the alteration of tissue function. It was reported that AGEs enhanced apoptosis in retinal pericytes, corneal endothelial cells, neuronal cells, and renal mesangial cells.[11–12,38] A major way in which AGEs exert their cellular effects is generally mediated by interaction with RAGE.[39] The interaction of AGEs and RAGE induced its downstream signalling and inflammatory response, including the production of cytokines and reactive oxygen species.[40] The accumulation of AGEs in retinal tissue increased oxidative stress and initiated a sequence of events that led to retinal cell apoptosis.[41,42] The oxidation of guanine to form 8-hydroxy-2′-deoxyguanosine serves as a marker of oxidative DNA damage.[43] When DNA is damaged, cells initiate a response, such as DNA repair, cell cycle delay, or induction of apoptosis.[44] Thus, the inhibition of AGE accumulation suggested novel therapeutic strategies for the treatment of diabetic corneal complication. Aminoguanidine, an AGE inhibitor, has been reported to attenuate the structural alterations of the diabetic cornea in treated animals.[30] Our previous studies showed that KIOM-79 had a stronger inhibitory effect on AGE formation *in vitro* than aminoguanidine, and it also reduced AGE accumulation in the

kidney and retina in type 1 and type 2 diabetic animal models.[18,19,32] KIOM-79 also had cellular antioxidant defence capacity through induction of haem oxygenase-1 in rat pancreatic beta-cells. At present, KIOM-79 reduced AGE accumulation and oxidative DNA damage in the cornea of ZDF rats.

Next, we examined whether KIOM-79 could inhibit the activation of NF-κB in diabetic corneas. NF-κB is a common downstream signal pathway of AGEs/RAGE axis.[45] It also plays an essential role in apoptosis through the release of pro-apoptotic molecules.[10,46] The binding of AGEs to RAGE leads to activation of NF-κB.[42] AGE-induced cellular oxidative stress also results in activation of NF-κB.[47] Thus, the activation of NF-κB is involved in sustained oxidative stress-related corneal damage. KIOM-79 had a preventative effect on up-regulated expression of activation of NF-κB in the diabetic cornea, consistent with the previous report that KIOM-79 blocked the activation of NF-κB in a lipopolysaccharide-activated macrophage cell.[20] These results suggested that KIOM-79 could prevent diabetic corneal damage by the inhibition of NF-κB activation. In addition, the gene expression level of Bax was increased by activated NF-κB in the retinal pericyte treated with high glucose.[10,48] The Bax promoter contains a potential binding site for NF-κB.[49] Moreover, the ratio of Bax to Bcl-2 was considered to be the principal factor in determining cell survival versus cell death.[50] In this study, KIOM-79 altered this ratio in favour of an anti-apoptotic response.

Four major compounds, magnolol, honokiol, glycyrrhizine and puerarin, were identified in KIOM-79.[51] Magnolol prevents oxidized low-density lipoprotein (oxLDL)-induced vascular endothelial apoptosis. Honokiol suppresses NF-κB activation and NF-κB-regulated gene expression through the inhibition of IkappaB kinases, and it attenuates oxLDL-induced apoptosis in vascular endothelial cells.[52,53] Glycyrrhizine inhibits 3-morpholinosydnonime-induced apoptosis in lung epithelial cells.[54] Puerarin has a protective effect against apoptosis in diabetic rat lens epithelial cells.[55] Therefore, the ability of KIOM-79 to prevent diabetes-induced corneal cell apoptosis may be a result of the activity of these compounds.

Conclusions

This experiment demonstrated that KIOM-79, which is a combination of extracts obtained from *M. officinalis*, *P. lobata*, *G. uralensis* and *E. pekinensis*, successfully inhibited the histological alterations and apoptosis of corneal cells. Based on our findings, the most likely mechanism for this biological effect involved the inhibition of oxidative DNA damage and NF-κB activation through the suppression of AGE accumulation.

Declarations

Conflict of interest

The Authors declare that there is no conflict of interest to disclose.

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