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Curcumin Alleviates Diabetic Retinopathy in Experimental Diabetic Rats

Fang Yang[a] Jinqiang Yu[a] Feng Ke[a] Mei Lan[a] Dekun Li[a] Ke Tan[a]
Jiaojiao Ling[a] Ying Wang[a] Kaili Wu[b] Dai Li[c]

[a]Department of Ophthalmology, Renmin Hospital, Hubei University of Medicine, Shiyan, China; [b]State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou, China; [c]Xianning Aier Eye Hospital, Hubei University of Science and Technology, Xianning, China

Keywords
Curcumin · Diabetic retinopathy · Ultrastructure · Vascular endothelial growth factor · Malondialdehyde · Superoxide dismutase · Total antioxidant capacity · Bax · Bcl-2

Abstract
Purpose: To investigate the potential protective effects of curcumin on the retina in diabetic rats. ***Methods:*** An experimental diabetic rat model was induced by a low dose of streptozotocin combined with a high-energy diet. Rats which had blood glucose levels ≥11.6 mmol/L were used as diabetic rats. The diabetic rats were randomly divided into 3 groups: diabetic rats with no treatment (DM), diabetic rats treated with 100 mg/kg curcumin (DM + Cur 100 mg/kg), and diabetic rats treated with 200 mg/kg curcumin (DM + Cur 200 mg/kg). Curcumin was orally administered daily for 16 weeks. After 16 weeks of administration, the rats were euthanized, and eyes were dissected. Retinal histology was examined, and the thickness of the retina was measured. Ultrastructural changes of retinal ganglion cells, inner layer cells, retinal capillary, and membranous disks were observed by electron microscopy. Malondialdehyde, superoxide dismutase, and total antioxidant capacity were measured by ELISA. Expression levels of vascular endothelial growth factor (VEGF) in retina tissues were examined by immunohistochemical staining and ELISA. Expression levels of Bax and Bcl-2 in retina tissues were determined by immunohistochemical staining and Western blotting. ***Results:*** Curcumin reduced the blood glucose levels of diabetic rats and decreased diabetes-induced body weight loss. Curcumin prevented attenuation of the retina in diabetic rats and ameliorated diabetes-induced ultrastructure changes of the retina, including thinning of the retina, apoptosis of the retinal ganglion cells and inner nuclear layer cells, thickening of retinal capillary basement membrane and disturbance of photoreceptor cell membranous disks. We also found that curcumin has a strong antioxidative ability in the retina of diabetic rats. It was observed that curcumin attenuated the expression of VEGF in the retina of diabetic rats. We also discovered that curcumin had an antiapoptotic effect by upregulating the expression of Bcl-2 and downregulating the expression of Bax in the retina of diabetic rats. ***Conclusions:*** Taken together, these results suggest that curcumin may have great therapeutic potential in the treatment of diabetic retinopathy which could be attributed to the hypoglycemic, antioxidant, VEGF-downregulating and neuroprotection properties of curcumin.

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E-Mail karger@karger.com
www.karger.com/ore

Dai Li
Xianning Aier Eye Hospital
Hubei University of Science and Technology
Xianning, Hubei 437000 (China)
E-Mail lidai9881@163.com

Introduction

Diabetic retinopathy (DR), one of the most common complications of diabetes mellitus (DM), is one of the major causes of vision loss all over the world. Almost all patients with diabetes suffer different degrees of retinopathy after 20 years' duration of diabetes [1]. It has long been believed that DR is a microvascular complication associated with endothelial dysfunction, which is characterized by pericyte and endothelial cell loss, capillary basement membrane thickening, blood-retina barrier leakage, and neovascularization [2–4]. In recent years, an increasing body of evidence has suggested that neuronal cell death of the retina is a critical component of DR [2, 4, 5]. In addition, neurodegeneration and innate immunity/sterile inflammation occur early in DR, preceding retinal vascular complications in both humans and experimental animals [2, 5, 6]. Furthermore, all types of retinal cells are affected including retinal ganglion cells (RGCs). As reported, ganglion cells in diabetic retinas express several proapoptotic molecules, such as caspase-3, Fas, and Bax, suggesting that these cells are the most vulnerable population in DR [5]. El-Remessy et al. [7] reported that neurotoxicity causes permanent impairment of visual function due to cell death of the inner retina and ganglion cells. Vascular endothelial growth factor (VEGF) is the most prominent member of a group of factors that control and facilitate physiological and pathological angiogenesis [3]. In patients with active proliferative DR, VEGF levels are increased, while in those eyes in which proliferative retinopathy is quiescent, VEGF levels are either normal or only modestly increased [8]. It was demonstrated that oxidative stress induced by hyperglycemia leads to oxidative injury of neurons in several studies, which then activates the death pathways implicated in neuronal apoptosis [9, 10]. Retinas from diabetic rats showed increased oxidative stress, and the administration of antioxidants inhibited the development of retinopathy [11]. Recently, many pro-oxidant proteins have been shown to be highly upregulated in DR and by high glucose in retinal cells in culture [4].

Because of the complicated pathogenesis of DR, drugs such as inhibitors for signaling pathways and growth factors were shown to be effective for the treatment of DR. Currently, intravitreal injection of anti-VEGF and corticosteroids are popular therapeutics but still have some limitations. A substantial proportion of patients do not respond to these therapies [12]. This suggests that some other factors or pathways independent of VEGF are involved in the development of DR. So there is an urgent need to find a positive and effective treatment for DR.

Traditional Chinese Medicine has been widely used for centuries and can play a unique therapeutic role in the treatment of many human diseases. Curcumin, a yellow curry pigment isolated from turmeric powder, has been reported to have a wide range of pharmacological effects, such as antioxidant [13–17], anti-inflammatory [13–15, 17, 18], antitumor [18, 19], scavenging reactive oxidative species [18], antiangiogenesis [18], modulating apoptosis [14, 18, 20], neuroprotective [21], inhibiting proliferation [22], antihyperglycemic [23], and so on. Curcumin has been used to treat DM in many Asian countries like China and India for a long time. Maithilikarpagaselvi et al. [15] found that curcumin attenuated glucose intolerance and insulin resistance through its antioxidant and anti-inflammatory effects and suggested the use of curcumin as a therapeutic adjuvant in the management of diabetes, obesity, and their associated complications. Curcumin also has protective effects against diabetic nephropathy [16]. However, the possibility whether curcumin may have beneficial effects in the treatment for DR or not has little to be addressed up to now.

In our present study, an experimental diabetic animal model was induced by a low dose of streptozotocin (STZ) combined with high energy intake in rats [24]. The aim of this study is to investigate the potential effects of curcumin on improving DR in diabetic rats, and the associated mechanisms as well. To test this hypothesis, we examined whether curcumin has hypoglycemic, neuroprotective, antioxidant, and VEGF-downregulating effects on the retina in the diabetic rats.

Materials and Methods

Experimental Animals

Seventy-five male Wistar rats (70–90 g) were purchased from Laboratory Animal Center (Hunan, China). Animals were housed in a specific pathogen-free animal house according to the guidelines established by the Association for Research in Vision and Ophthalmology's Statement for the Use of Animals in Ophthalmic and Vision Research. Before experiments, all rats were fed a basal diet for 1 week. They were housed with a 12-h light/dark cycle at a temperature of 23–25 °C and humidity of 55–60%.

Induction of Experimental Diabetes on Rats

Sixty-five rats were made diabetic by feeding a high-fat diet during the whole experimental period, whereas the other 10 rats consuming the basal diet at the same time served as a control group. The high-fat diet was prepared by adding 20% sucrose (w/w) and 20% lard (w/w) to the basal diet [24]. After 8 weeks, rats

Table 1. Reagents used in this study

Products	Product No.	Manufacturer
SOD (ELISA)	A003-2	Nanking Jiancheng Bioengineering Research Institute, China
MDA (ELISA)	A001-3	Nanking Jiancheng Bioengineering Research Institute, China
T-AOC (ELISA)	A015	Nanking Jiancheng Bioengineering Research Institute, China
Mouse anti-VEGF (IHC)	sc-57496	Santa Cruz, CA, USA
Mouse anti-Bax (IHC)	sc-493	Santa Cruz, CA, USA
Mouse anti-Bcl-2 (IHC)	sc-7382	Santa Cruz, CA, USA
Mouse Anti-Bax (WB)	#2772S	Cell Signaling Technology, Boston, MA, USA
Mouse Anti-Bcl-2 (WB)	#2876	Cell Signaling Technology, Boston, MA, USA
Mouse Anti-β-actin (WB)	sc-58673	Santa Cruz Biotechnology, Texas, TX, USA

SOD, superoxide dismutase; ELISA, enzyme-linked immunosorbent assay; MDA, malondialdehyde; T-AOC, total antioxidant capacity; VEGF, vascular endothelial growth factor; IHC, immunohistochemistry; WB, Western blot.

fed with the high-fat diet were intraperitoneally injected just once with STZ (Sigma Aldrich, St. Louis, MO, USA) at the dose of 40 mg/kg dissolved in 100 mM citrate buffer, pH 4.5 [24]. Ten rats which served as controls were given the same volume of citrate buffer instead of STZ. Blood glucose levels were measured 72 h after STZ injection using a hand-held glucometer (Changsha Sinocare Inc.) by tail vein puncture blood sampling. Forty-four rats with blood glucose values ≥11.6 mmol/L were considered diabetic. One-week diabetic rats were treated with curcumin (Cur; 100 or 200 mg/kg/day p.o. [23]) for 16 weeks. Curcumin (Shaanxi Sciphar Natural Products Co. Ltd., China) was diluted in carboxymethyl cellulose sodium. The rats in the control and diabetic groups were administered equivalent volumes of carboxymethyl cellulose sodium. All the animals were provided with food and water ad libitum. Body weight was recorded every week. After 16 weeks of curcumin administration, the rats were euthanized by intraperitoneal injection of 1% pentobarbital (50 mg/kg), and the eyes were dissected. Four eyes from 4 rats in each group were immediately fixed in 4% paraformaldehyde solution for histopathological examination. Four retinas from 4 individuals in each group were immediately placed in a 2.5% glutaraldehyde solution for ultrastructural analysis. The remaining retinas were carefully dissected under a stereomicroscope, then frozen immediately in liquid nitrogen, and stored at –80 °C for Western blotting and ELISA analysis.

Hematoxylin-Eosin Staining and Morphology Examination

Four paraformaldehyde-fixed eye tissues from each group were embedded in paraffin and sectioned at 5 μm thickness each for hematoxylin-eosin (HE) staining and immunohistochemical staining of VEGF, Bax and Bcl-2.

All color micrographs were collected using a microscope and digital camera (Axioplan 2 imaging; Carl Zeiss Inc., Shanghai, China; camera equipped with Spot Software ver. Axiovision Rel 4.8). Measurements of the thickness of the total retina were determined by the Spot Software ver. Axiovision Rel 4.8. The thickness was measured on the magnified images (×400) of the retinal equator at 10 points: 5 on either side of the retinal optic nerve, located approximately 50–60 μm apart from the optic nerve. The mean thickness value of the 4 eyes was recorded as the representative value for each group. The results of the 4 groups were analyzed by ANOVA.

Transmission Electron Microscopy Detection

Four pieces of retina approximately 2 mm × 3 mm in size were isolated from each eyecup, approximately 2 mm from the optic disk, following the protocol described previously [25]. Briefly, retina tissues were immediately prefixed in 2.5% glutaraldehyde for 2 h and then fixed in 1% osmium tetroxide. Sequentially, the tissues were dehydrated and embedded in Epon 812. The tissues were sectioned at a thickness of 50–60 nm using an ultramicrotome and double-stained with uranyl acetate and lead citrate. The ultrastructure of the retina tissues was examined using a transmission electron microscope (TEM H-600, Hitachi, Tokyo, Japan).

Estimation of Superoxide Dismutase, Malondialdehyde, Total Antioxidant Capacity, and VEGF with ELISA in Retina

The samples of retina tissue were weighed and homogenized (1:10, w/v) in 50 mmol/L phosphate buffer (pH 7.4). The superoxide dismutase (SOD), malondialdehyde (MDA), total antioxidant capacity (T-AOC), and VEGF (A003-2, A001-3, A015, Nanking Jiancheng Bioengineering Research Institute, China) levels were measured by colorimetric analysis using a spectrophotometer with the associated detection kits (Table 1).

Immunohistochemical Determination of VEGF, Bax, and Bcl-2

To examine VEGF, Bax, and Bcl-2 protein expression in the retinal tissue, immunohistochemical staining was performed on paraffin sections as previously reported [26]. Four sections of each paraffin-embedded eye tissue from each group were applied for immunohistochemical staining. Tissue sections were incubated with polyclonal primary antibodies against mouse anti-VEGF, mouse anti-Bax or mouse anti-Bcl-2 (sc-57496, sc-493, sc-7382; Santa Cruz Biotechnologies, Santa Cruz, CA, USA) overnight at 4 °C. After washing with phosphate-buffered saline, the sections were incubated with biotinylated horse anti-mouse IgG (Invitrogen) for 30 min and then incubated with the avidin-biotin-peroxidase complex using an ABC kit. The reaction was visualized by color development with 3,3′-diaminobenzidine tetrahydrochloride. All sections were counterstained with hematoxylin. Images

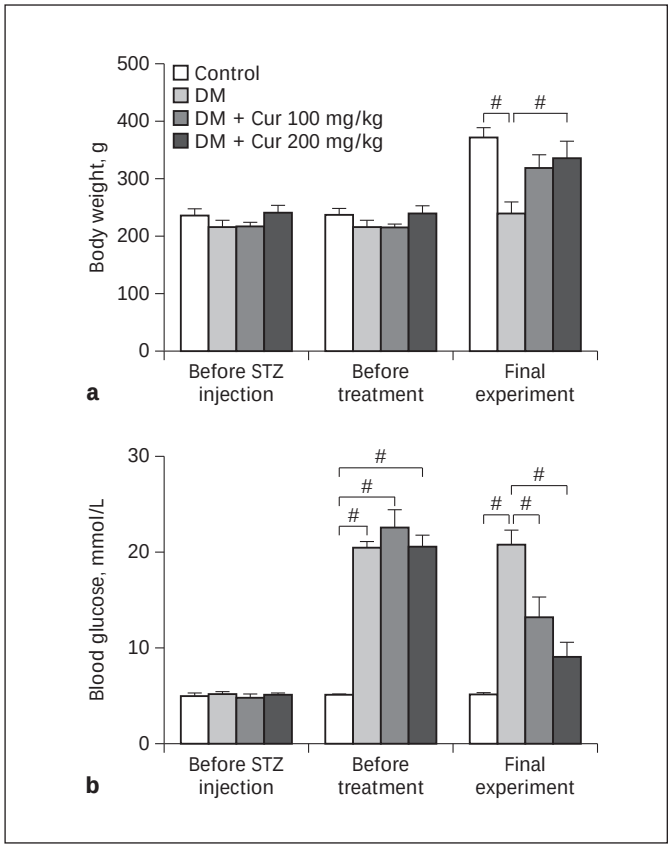

Fig. 1. Effect of curcumin (Cur) on body weight and blood glucose levels of diabetic rats at the important time points. DM, diabetes mellitus. **a** The body weight in each group before streptozotocin (STZ) injection (after 8 weeks feeding from the beginning), before curcumin treatment (1 week after STZ injection), and at the end of the experiment. Sixteen-week treatments with curcumin at the dose of 200 mg/kg increased body weight of the diabetic rats compared with the untreated diabetic group ($p < 0.01$). **b** The blood glucose level in each group before STZ injection (after 8 weeks feeding from the beginning), before curcumin treatment (1 week after STZ injection), and at the end of the experiment. At the end of the experiment, 16-week treatments with curcumin at the doses of 100 and 200 mg/kg decreased blood glucose of the diabetic rats compared with the untreated diabetic group ($p < 0.01$). Data are expressed as means ± standard deviation. $^{\#}$ $p < 0.01$.

from the immunohistochemical studies of VEGF, Bax, and Bcl-2 protein expression (×400) were photographed with a microscope and digital camera (Axioplan 2 imaging; Carl Zeiss Inc., Shanghai, China; the camera was equipped with Spot Software ver. Axiovision Rel 4.8).

Western Blot Analysis of Bax and Bcl-2

Western blot tests were performed as described previously [27]. The retinas in each group were pooled and homogenized in ice-cold lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 μg/mL aprotinin, leupeptin and pepstatin, and 1 mM phenylmethylsulfonyl fluoride) and centrifuged at 12,890 *g* for 15 min at 4 °C. The supernatant was collected, and the protein concentration was measured using the bicinchoninic acid protein assay (Beyotime, Jiangsu, China). An equal amount of protein for each sample (60 μg/lane) was separated on 10% SDS-polyacrylamide gel and transferred to a polyvinylidene fluoride membrane. The membranes were blocked in 5% skimmed milk for 2 h at room temperature before incubation with an antibody against Bax, Bcl-2 (Cell Signaling Technology, Boston, MA, USA), or β-actin (Santa Cruz Biotechnology, Texas, TX, USA) overnight at 4 °C. The membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (GE Healthcare, UK) for 2 h, and blots were developed using an ECL kit (Pierce Biosciences, Rockford, IL, USA). The expression of β-actin was used as an internal loading control to standardize the amount of loaded protein. Experiments were repeated 5 times. The intensity of the protein band was semiquantitatively measured by image analysis software (Image-Pro plus 6.0, Media Cybernetics, Maryland, MD, USA).

Statistical Analysis

All data were reported as the mean ± standard deviation. One-way ANOVA and Student-Newman-Keuls tests were performed to compare the means of the groups using statistical software (SPSS statistics 17.0, New York, NY, USA). A value of $p < 0.05$ was considered statistically significant.

Results

Curcumin Inhibits Diabetes-Induced Body Weight Loss and Reduces Blood Glucose of Diabetic Rats

Figure 1 shows the body weight and blood glucose levels of each group at the important time points in this study.

The body weight in each group was not significantly different before STZ injection (after 8 weeks feeding from the beginning) and before curcumin treatment (1 week after STZ injection). At the end of the experiment, untreated diabetic rats had markedly lower body weights compared with the control group ($p < 0.01$); however, 16-week treatments with curcumin at the dose of 200 mg/kg increased body weight of the diabetic rats compared with the untreated diabetic rats ($p < 0.01$).

There was no significant difference of blood glucose between all groups before STZ injection. One week after STZ injection, the blood glucose level of the rats in the DM groups was higher than that of the controls ($p < 0.01$). At the end of the experiment, as shown in Figure 1, blood glucose levels of diabetic rats significantly increased compared to control rats ($p < 0.01$). Sixteen-week curcumin at the dose of 100 mg/kg decreased blood glucose of the

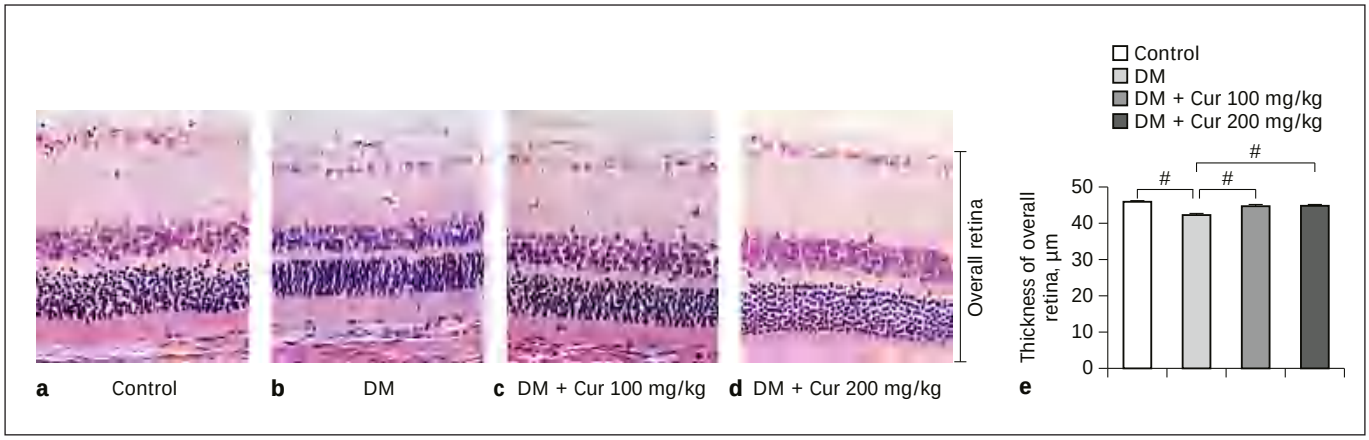

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Fig. 2. Effect of curcumin (Cur) on the morphology of the retinas in diabetic rats. DM, diabetes mellitus. **a–d** Representative images of HE-stained sections of retinas of the rats. Magnification ×400. **b–d** The thickness of the overall retina in the diabetic rats was reduced by 7.4% compared with the control group. **e** In diabetic rats treated with curcumin (DM + Cur 100 mg/kg and DM + Cur 200 mg/kg), the thickness of the overall retina increased compared with the diabetic group. Data are expressed as means ± standard deviation. $^{\#}$ $p < 0.01$.

diabetic rats compared with the untreated diabetic rats ($p < 0.01$). Meanwhile, compared with untreated diabetic rats, 16-week curcumin at the dose of 200 mg/kg decreased blood glucose of the diabetic rats too ($p < 0.01$).

Curcumin Prevents Attenuation of the Retina of Diabetic Rats

Systematic morphology examination of HE-stained retinal sections (Fig. 2) showed that the overall thickness of the retina in the diabetic rats was reduced by 7.4% compared with the control group (42.13 ± 0.49 vs. 45.49 ± 0.60 μm, $p < 0.01$). In diabetic rats treated with curcumin (DM + Cur 100 mg/kg and DM + Cur 200 mg/kg), the retina appeared much more normal (Fig. 2a), and the thickness of the overall retina increased compared with the diabetic group (44.60 ± 0.53 and 44.75 ±0.35 vs. 42.13 ± 0.49 μm, $p < 0.01$).

Curcumin Ameliorates Diabetes-Induced Ultrastructure Change of Retina

Retinal ultrathin sections were systematically examined by transmission electron microscopy to determine whether curcumin can ameliorate apoptosis of the RGCs and inner nuclear layer (INL) cells, thickening of the retinal capillary basement membrane, and disturbance of photoreceptor cell membranous disks.

RGCs in normal control rats had uniformly normal-appearing nuclei and mitochondria (Fig. 3a). An apoptotic body, a morphological characteristic of apoptosis, was detected in the extracellular matrix of RGCs in diabetic rats (Fig. 3e). Meanwhile, vacuolation, margination of chromatin, and swollen mitochondria were discovered in RGCs of diabetic rats (Fig. 3e), while the RGCs in diabetic rats treated with 100 and 200 mg/kg curcumin did not show obvious vacuolation and swollen mitochondria (Fig. 3i, m).

INL cells in control rats looked normal in appearance (Fig. 3b). There were abundant vacuolation and margination of chromatin discovered in INL cells of diabetic rats (Fig. 3f), while these cells in diabetic rats treated with 100 and 200 mg/kg curcumin showed less vacuolation and margination of chromatin than diabetic rats (Fig. 3j, n).

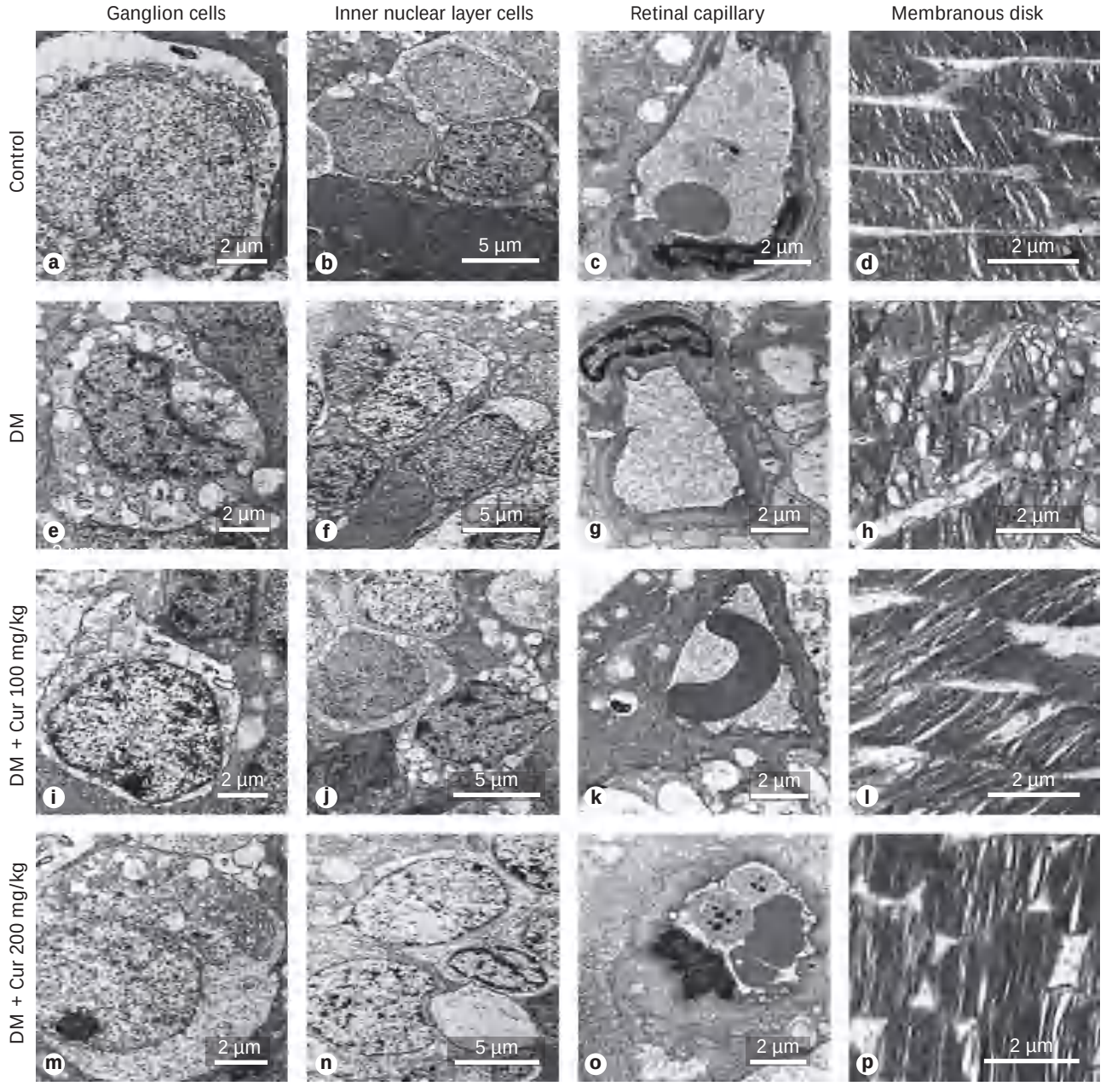
An electron microscopy analysis of the retinal capillary (Fig. 3c–o) showed a significant increase in retinal capillary basement membrane thickness in the diabetic rats compared to those of normal control rats. Upon treatment with curcumin, a significant reduction in retinal capillary basement membrane thickness was observed in the retinas of diabetic rats compared to those of untreated diabetic model rats.

The photoreceptor cell membranous disks produce orderliness, and their structure was discernible in control rats (Fig. 3d). In diabetic rats, the photoreceptor cell membranous disks appeared inordinate, frizzy, loose, and swollen (Fig. 3h). Nevertheless, curcumin at the dose of 100 and 200 mg/kg improved the structure disturbance of photoreceptor cell membranous disks (Fig. 3l, p).

Curcumin Attenuates the Expression of VEGF in Retinas of Diabetic Rats

VEGF is the most prominent member of a group of factors that facilitate pathological angiogenesis in diabetes. The expression of VEGF was assessed by immunohistochemistry (Fig. 4a–d) and ELISA (Fig. 4e).

As shown in Figure 4a–d, by immunohistochemistry staining representative images of each group, the expression of VEGF was detected and mainly localized in the cytosol of cells in the ganglion cell layer (GCL) and INL (Fig. 4b, c, black arrows).The expression of VEGF was increased in retinas of diabetic rats compared to control rats ($p < 0.01$). Remarkably, diabetic rats treated with 100 and 200 mg/kg curcumin could significantly downregulate VEGF expression ($p < 0.01$).



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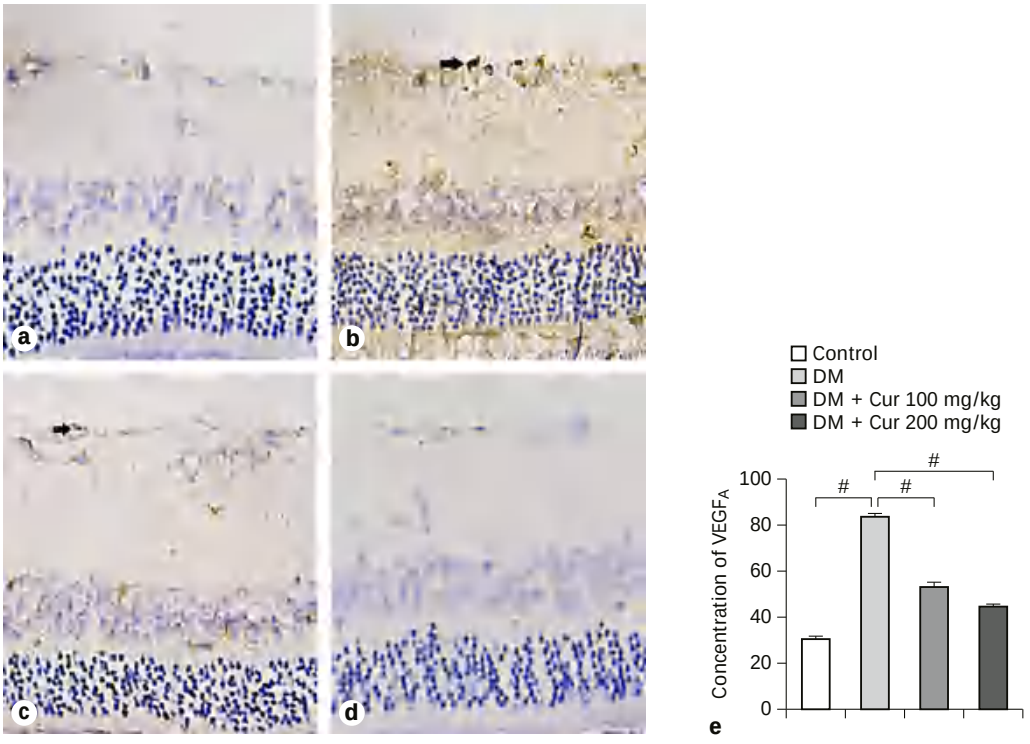

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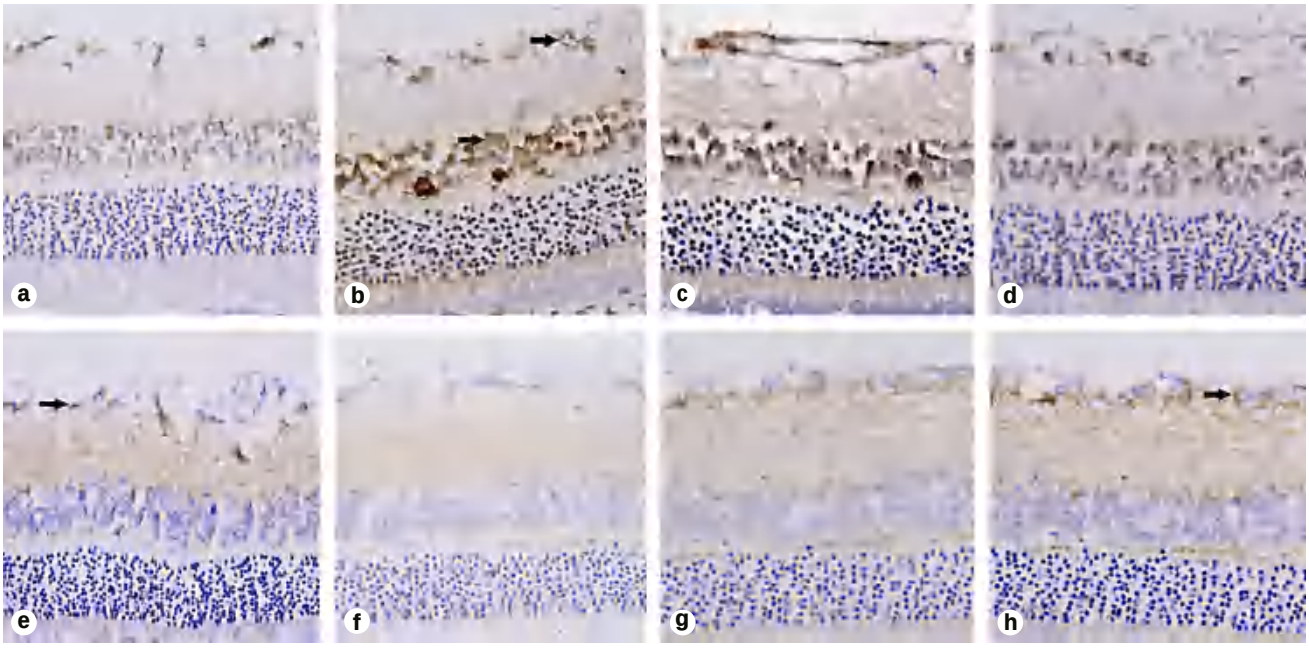
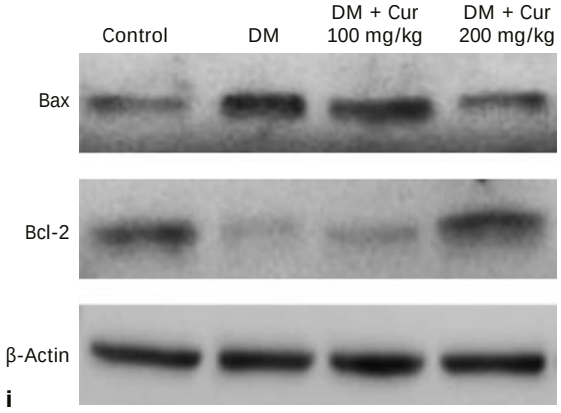
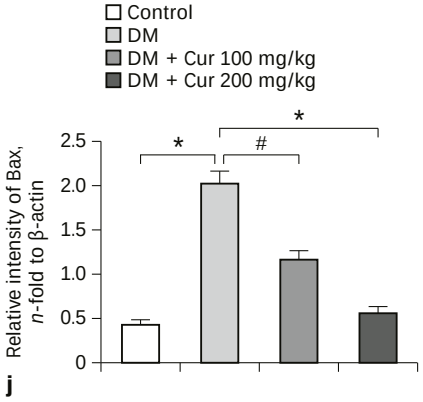
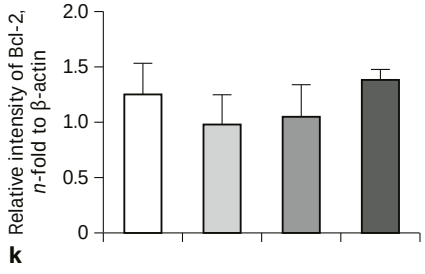
Fig. 4. Effect of curcumin (Cur) on the expression of VEGF in retinas of diabetic rats. Vascular endothelial growth factor (VEGF) expression was assessed by immunohistochemistry (**a–d**) and ELISA (**e**). **a–d** Immunohistochemistry staining representative images of each group. The expression of VEGF was detected and mainly localized in the cytosol of cells in the ganglion cell layer and inner nuclear layer (black arrows). **e** The concentration of VEGF was assessed by ELISA. VEGF concentration was increased in retinas of diabetic rats compared to control rats ($p < 0.01$). Remarkably, diabetic rats treated with 100 and 200 mg/kg curcumin could significantly downregulate VEGF expression ($p < 0.01$). Data are expressed as means ± standard deviation. $^{\#}$ $p < 0.01$.

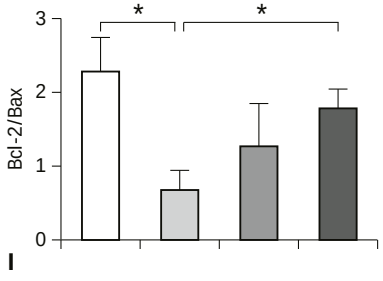
Fig. 3. Effect of curcumin (Cur) on diabetes-induced ultrastructure change of retina. $n = 4$ per group. Five electrophotographs of each group were analyzed. DM, diabetes mellitus. **a**, **e**, **i**, **m** Representative images showing the ultrastructural changes of retinal ganglion cells (RGCs) of each group. Scale bar = 2 μm. **b**, **f**, **j**, **n** Representative images which show the ultrastructural changes of retinal inner nuclear layer cells of each group. Scale bar = 5 μm. **c**, **g**, **k**, **o** Representative ultramicroscopic images of retinal capillary of each group (white arrows show the retinal capillary basement membrane). Scale bar = 2 μm. **d**, **h**, **l**, **p** Representative images exhibiting the ultrastructural changes of photoreceptor cell membranous disks in each group. Scale bar = 2 μm. **a** RGCs in normal control rats had uniformly normal-appearing nuclei and mitochondria. **e** An apoptotic body, a morphological characteristic of apoptosis, was detected in the extracellular matrix of RGCs in diabetic rats. Meanwhile, vacuolation, margination of chromatin, and swollen mitochondria were discovered in RGCs of diabetic rats. **i**, **m** RGCs in diabetic rats treated with 100 and 200 mg/kg curcumin showed some vacuolation and swollen mitochondria. **b** Inner nuclear layer cells in control rats look normal. **f** There were abundant vacuolation and margination of chromatin discovered in inner nuclear layer cells of diabetic rats. **j** In diabetic rats treated with 100 mg/kg curcumin, there were still some vacuolation and margination of chromatin detected in inner nuclear layer cells. **n** A small quantity of vacuolation and margination of chromatin was detected in inner nuclear layer cells in diabetic rats treated with 200 mg/kg curcumin. **c** Normal retinal capillary of control rats. **g** A significant increase in retinal capillary basement membrane thickness was discovered in diabetic rats. **k**, **o** Upon treatment with curcumin at the dose of 100 and 200 mg/kg, a significant reduction in retinal capillary basement membrane thickness was observed in the retinas of diabetic rats compared to those of treated diabetic model rats. **d** The photoreceptor cell membranous disks show orderliness, and their structure is discernible in control rats. **h** In diabetic rats, the photoreceptor cell membranous disks appeared inordinate, frizzy, loose and swollen. **l**, **p** Curcumin at the dose of 100 and 200 mg/kg improved the structure disturbance of photoreceptor cell membranous disks.











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Curcumin Inhibits Oxidative Stress in Retinas of Diabetic Rats

MDA is one of the most important products of membrane lipid peroxidation. SOD is the primary enzyme that removes oxygen free radicals in vivo. T-AOC can reflect the antioxidant capacity of an organism.

As shown in Table 2, there was increased accumulation of lipid peroxides with a concordant increased content of MDA and a decreased activity of SOD and T-AOC in retinas of DM group rats ($p < 0.01$ vs. control group). The treatment of diabetic rats with 200 mg/kg curcumin obviously reduced the MDA content and upregulated the activity of SOD and T-AOC ($p < 0.01$ vs. DM group).

Curcumin Accommodates the Expression of Bax and Bcl-2 in Retinas of Diabetic Rats

The expressions of the proapoptotic protein Bax and the antiapoptotic protein Bcl-2 were examined using immunohistochemistry staining and Western blotting (Fig. 5). Immunohistochemistry staining (Fig. 5a–h) showed that Bax- and Bcl-2-positive signals were localized in the cytosol of cells in the GCL and INL. Both immunohistochemistry and Western blot analysis (Fig. 5i–k) revealed that an enhanced expression of Bax but a relatively reduced expression of Bcl-2 was presented in the diabetic group in contrast to the control group. However, treatments with 100 and 200 mg/kg curcumin decreased Bax expression compared to the diabetic group. There was no significant difference in relative intensities of Bcl-2 between the groups. As shown in Figure 5l, the expression ratio of Bcl-2 to Bax was significantly decreased in diabetic rat retinas compared to the control group ($p < 0.01$). In contrast, curcumin at the dose of 200 mg/kg enhanced that ratio of Bcl-2 to Bax compared to the diabetic group ($p < 0.01$). Thus, the ratio of Bcl-2 to Bax demonstrated the ability of curcumin to inhibit apoptosis of the retina by upregulating the expression of Bcl-2 and downregulating that of Bax.

Table 2. Effect of curcumin (Cur) on malondialdehyde (MDA), superoxide dismutase (SOD), and total antioxidant capacity (T-AOC) levels of diabetic rats

Group	MDA, nmol/mg protein	SOD, U/mg protein	T-AOC, U/mg protein
Control	0.044±0.001	22.47±1.10	32.90±1.25
DM	0.071±0.004[a]	11.32±0.55[a]	13.46±0.43[a]
DM + Cur 100 mg/kg	0.058±0.003	17.87±0.73[b]	16.30±0.59[b]

Data are expressed as means ± standard deviation. [a] $p < 0.01$ versus control group; [b] $p < 0.01$ versus diabetes mellitus (DM) group.

Discussion

This present study investigated the protective effects of curcumin in preventing retinal damage in STZ-induced diabetic rats. Curcumin reduced the blood glucose levels of diabetic rats and decreased diabetes-induced body weight loss in this study. Curcumin prevented the attenuation of the retina of diabetic rats and ameliorated any diabetes-induced ultrastructure change of the retina, including thinning of the retina, apoptosis of the RGCs and INL cells, thickening of the retinal capillary basement membrane and disturbance of photoreceptor cell membranous disks. We also found that curcumin has a strong antioxidative ability in the retina of diabetic rats. It was observed that curcumin attenuated the expression of VEGF in the retina of diabetic rats. We also discovered that curcumin demonstrated an antiapoptotic effect by upregulating the expression of Bcl-2 and downregulating the expression of Bax in the retina of diabetic rats.

Curcumin, the main active ingredient of turmeric and the most abundant polyphenol present in the dietary spice turmeric, has a wide range of pharmacological effects. Many reports considered curcumin to be an effective antioxidant against oxidative damage [13–17]. Curcumin has been shown to exert anti-inflammatory effects in various acute and chronic diseases such as hepatic diseases [13], Huntington disease [14], diabetes [15, 17], car-

Fig. 5. Effect of curcumin (Cur) on the expression of apoptosis-related proteins Bax and Bcl-2 in retinas of diabetic rats. **a–h** Immunohistochemistry staining representative images of each group. Bax- and Bcl-2-positive signals were localized in the cytosol of cells in the ganglion cell layer and inner nuclear layer (black arrows). **i–k** Western blot analyses of the expression of Bax and Bcl-2. Enhanced expression of Bax but relatively reduced expression of Bcl-2 is presented in the diabetic group in contrast to the control group. However, 100 and 200 mg/kg curcumin treatment decreased Bax expression compared to the diabetic group. There was no significant difference of relative intensities of Bcl-2 between the groups. **l** The ratio of Bcl-2 to Bax expression was significantly decreased in diabetic retinas compared to the control group. In contrast, curcumin at the dose of 200 mg/kg enhanced that ratio compared to the diabetic group. Data are expressed as means ± standard deviation. * $p < 0.01$; # $p < 0.05$.

diovascular disease [17], cancer [18], and so on. Curcumin exerts its chemopreventive effects by promoting tumor apoptosis, inhibiting proliferation and antiangiogenesis, scavenging reactive oxidative species, and reducing the inflammatory molecules in cancer cells [18]. In Chongtham and Agrawal's study [14], they provided evidence that curcumin significantly ameliorates disease symptoms in a *Drosophila* model of Huntington disease by suppressing cell death and that it can be a key to halting the progression of Huntington disease with the least side effects. In unilateral ureteral obstruction in rats, the cytoprotective role of curcumin relies on its ability to decrease the TNFR2 mRNA and enhance the antiapoptotic molecules RIP and TRAF2 to decrease the apoptotic pathway via decreasing caspase 8 [28]. It is clear that curcumin has the ability to enhance neuronal survival, reduce apoptotic changes, and thereby produce neuroprotective effects [21]. Maithilikarpagaselvi et al. [15] found that curcumin attenuated glucose intolerance and insulin resistance through its antioxidant and anti-inflammatory effects in fructose-induced insulin-resistant animal models and suggested the use of curcumin as a novel therapeutic adjuvant in the management of diabetes, obesity, and their associated complications. Several studies have demonstrated that curcumin is atoxic, although in very high doses. Treatment of humans for 3 months with 8,000 mg curcumin per day showed no side effects [29]. In recent years, the interest for curcumin in the prevention and treatment of diabetes and associated complications has increased. We hypothesized that curcumin can exert protective effects on the retina of diabetic rats. We also investigated the protective effect of curcumin on the retina in diabetic rats and the potential mechanism involved.

Experimental diabetes produced by low doses of STZ combined with high energy intake is regarded as a general strategy to obtain animal models of type 2 diabetes, since it simulates the real course of human type 2 diabetes mellitus [24]. The high energy diet induces insulin resistance at first, and then an injection of low-dose STZ causes a partial dysfunction of the beta cells to suppress insulin secretion, which works as a compensation to insulin resistance.

The pathogenesis of DR is complex and is not a consequence of a single mechanism. Initially, DR was considered a microvascular complication of endothelial dysfunction, as it is characterized by capillary basement membrane thickening, pericyte and endothelial cell loss, blood-retina barrier breakdown and leakage, acellular capillaries, and neovascularization [2, 5]. However, it is currently acknowledged that before the typical features of DR occur, many types of retinal cells are affected with molecular and functional changes including ganglion cells [2, 5, 30]. Also, thinning of the retina [2, 31], changes in neurocyte morphology [31, 32], apoptosis of retinal neurons [31], and retinal pigment epithelium dysfunction [30] occur in DR and result in the gradual loss of retinal function. Therefore, DR is not only a vascular disease, but also a neurodegenerative one. In this study, systematic morphology examination of HE-stained retinal sections showed that the overall thickness of the retina in the diabetic rats was reduced compared with the control group, while, in the curcumin-treated diabetic rat group, the thickness of the overall retina increased compared with the diabetic group. By systematic transmission electron microscopy, morphological characteristics of apoptosis were detected in RGCs and INL cells in diabetic rats. It showed a significant increase in retinal capillary basement membrane thickness in the diabetic rats compared to those of normal control rats, and the photoreceptor cell membranous disks appeared inordinate, frizzy, loose, and swollen in diabetic rats. However, in the present study, it was discovered that curcumin can ameliorate apoptosis of the RGCs and INL cells, thickening of retinal capillary basement membrane, and disturbance of photoreceptor cell membranous disks.

VEGF was believed to be the most important intermediary in neovascularization and permeability of the diabetic retina. VEGF elevation induces a decrease in the tight-junction proteins and breakdown of the blood-retina barrier [33], an increase in leukostasis within retinal vessels [34], inflammation [35, 36], upregulation of intercellular adhesion molecule-1 expression [37], an increase in all nitric oxide synthase isoforms [37], and a metabolic imbalance in inorganic phosphate [38], all of which have been reported to contribute to DR pathology. The expression of VEGF was assessed by immunohistochemistry and ELISA in this study. The expression of VEGF was detected and mainly localized in the cytosol of cells in the GCL and INL. The expression of VEGF was increased in retinas of diabetic rats compared to control rats as described before. Remarkably, we found that diabetic rats treated with 100 and 200 mg/kg curcumin significantly downregulated VEGF expression. Gupta et al. [32] have also shown similar effects of curcumin on VEGF expression in the STZ-induced diabetic rat retina. But how does curcumin inhibit the expression of VEGF in the diabetic rat? We need to do more research about this issue.

Hyperglycemia and dyslipidemia in DM induced an increased lipid peroxidation, and peroxyl radical formation is an important mechanism in the genesis of micro-

angiopathy. Kumawat et al. [39] reported that MDA was significantly elevated in both diabetic groups whereas the antioxidant enzymes SOD, glutathione peroxidase, glutathione reductase, catalase, reduced glutathione, etc. were significantly decreased which might be helpful in the risk assessment of various complications of DM. In the study of Maithilikarpagaselvi et al. [15], treatment with curcumin inhibited the rise of MDA and the total oxidant status in the skeletal muscle of fructose-fed rats. In our present study, the same as in the previous report, there was increased accumulation of lipid peroxides with a concordantly increased content of MDA and activity of SOD and T-AOC in retinas of diabetic rats compared with control rats. Nevertheless, diabetic rat treatment with 200 mg/kg curcumin obviously reduced the MDA content and upregulated the activity of SOD and T-AOC. These results certified that curcumin has an antioxidant effect in the retina of diabetic rats.

Neuronal cell death of the retina is a critical component of DR. Increased apoptosis of neural retinal cells in experimental diabetes in rats and DM in humans has recently been documented. However, the molecular base of the apoptosis in diabetes in the retina is not yet identified. The molecular events regulating apoptosis are complex and involve genes that are both proapoptotic and antiapoptotic. A number of mediators are involved in apoptosis, including caspases, Fas/Fas ligand, Bax/Bcl-2, survivin, and p53 [5]. Ganglion cells in diabetic retinas expressed the apoptosis-promoting factors caspase-3, Fas, and Bax, showed upregulation of the antiapoptotic marker Bcl-2, and expressed the cytotoxic effector molecule Fas/Fas ligand [5]. Several reports indicate that Bax is a critical factor in retinal neuronal cell apoptosis and one of the targets for a therapeutic gene strategy to protect damaged retinal neurons [40, 41]. The intracellular Bcl-2 protein extended cell survival because it specifically blocked apoptotic cell death following a variety of signals [42]. The expression ratio of Bcl-2 to Bax is critical for the determination of the life span of cells [43]. In the present study, a significant increase in Bax protein expression was found in the retina of diabetic rats. The expression ratio of Bcl-2 to Bax clearly decreased, as it was implicated in the apoptosis of retinal neural cells in previous studies [31]. The administration of 100 and 200 mg/kg curcumin reduced the expression of the Bax protein and enhanced the ratio of Bcl-2 to Bax. It is possible that the curcumin-induced neuroprotection was mediated by normalizing the Bcl-2/Bax level, which contributed to the downregulation of Bax and the upregulation of Bcl-2 expression.

This study clearly demonstrates the therapeutic potential of curcumin in treating DR in diabetic rats. This protective effect of curcumin occurred in a dose-dependent manner. We demonstrated that the beneficial effects of curcumin against the progression of DR could be attributed to the hypoglycemic, antioxidant, VEGF-downregulating, and neuroprotective properties of curcumin. We concluded that curcumin could have potential benefits in the prevention of the onset and progression of retinopathy in diabetic patients. The protective effects of curcumin can be due to multiple beneficial effects on different retinal cells. Further investigations should be undertaken to clarify these beneficial effects.

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Disclosure Statement

We declare that no conflict of interest exists.

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