

## Research Article

# Vitamin D<sub>3</sub> Protects against Diabetic Retinopathy by Inhibiting High-Glucose-Induced Activation of the ROS/TXNIP/NLRP3 Inflammasome Pathway

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Received 17 July 2017; Revised 15 October 2017; Accepted 16 November 2017; Published 22 February 2018

Academic Editor: Amany Tawfik

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**Purpose.** This study aimed to evaluate the mechanisms underlying the effects of 1,25-dihydroxyvitamin D (vitamin D<sub>3</sub>) on diabetes-induced retinal vascular damage and retinal vascular endothelial cell apoptosis. **Methods.** Diabetic and control rats were randomly assigned to receive vitamin D<sub>3</sub> or vehicle for 6 months. Additionally, human retinal microvascular endothelial cells (HRMECs) were incubated in normal or high-glucose medium with or without vitamin D<sub>3</sub>. Morphological changes in retinal tissues and retinal vascular permeability were examined, and cellular apoptosis was detected by fluorescence staining. Intracellular reactive oxygen species (ROS) levels were determined using fluorescent probes. Proteins were examined by Western blotting. **Results.** Vitamin D<sub>3</sub> significantly downregulated intracellular ROS and inhibited TRX-interacting protein (TXNIP)/NOD-like receptor family, pyrin domain-containing 3 (NLRP3) inflammasome pathway activation. Additionally, vitamin D<sub>3</sub> reduced vascular endothelial growth factor (VEGF) expression and the Bax/Bcl-2 ratio. These changes were associated with retinal recovery and with decreases in retinal vascular permeability and retinal capillary cell apoptosis. **Conclusions.** Vitamin D<sub>3</sub> decreases diabetes-induced ROS and exerts protective effects against retinal vascular damage and cell apoptosis in association with inhibition of the ROS/TXNIP/NLRP3 inflammasome pathway. Understanding the mechanisms of action of vitamin D<sub>3</sub> has important implications for preventing and treating inflammatory-related illnesses such as diabetic retinopathy.

## 1. Introduction

The total number of patients with diabetes worldwide is expected to increase from 171 million in 2000 to 366 million by 2030 and constitutes one of the major threats to global health [1]. Regarding vascular complications of diabetes, 35% of individuals with diabetes have some form of diabetic retinopathy (DR), 7% have proliferative DR (PDR), 7% have diabetic macular edema, and 10% are in vision-threatening stages [2]. Therefore, identifying methods of inhibiting the development of DR has become a focus of investigation.

Vitamin D is a multifunctional hormone, as its activated metabolite, 1,25-dihydroxy vitamin D<sub>3</sub>, has many diverse biological functions in addition to its classical role in calcium and bone homeostasis, including blood pressure control, immunoregulation, apoptosis inhibition, and antiangiogenesis [3, 4]. Moreover, vitamin D<sub>3</sub> plays an important role in endothelial function and exerts antioxidant and anti-inflammatory effects [5–7]. Recently, several epidemiological and clinical studies demonstrated the association between vitamin D<sub>3</sub> and DR [8–12]. At the genetic level, several polymorphisms in the vitamin D receptor (VDR) are associated

with an increased risk of DR [13–16]. There is accumulating evidence that hypovitaminosis D is closely related to DR. Payne et al. reported that patients with diabetes, especially those with PDR, have lower 25(OH)D levels than those without diabetes [17]. Ahmadieh et al. found that a low serum 25(OH)D level was an independent predictor of DR in patients with diabetes mellitus type 2 (DM2). Further research has indicated that vitamin D prevents the development and progression of DR by inhibiting inflammation and neovascularization in retinal tissues [18]. Specifically, vitamin D might decrease the proliferation of immunocytes and the expression of proinflammatory cytokines and down-regulate the transcriptional activity of hypoxia-inducible factor-1 as well as its target genes, such as vascular endothelial growth factor (VEGF). However, Bonakdaran et al. reported in a clinic-based cross-sectional study that decreased serum vitamin D levels were not associated with an increased risk of DR or its risk factors. Thus, the mechanism by which vitamin D<sub>3</sub> inhibits DR remains to be elucidated.

Chronic inflammation underlies much of the pathology of DR [19]. Many physiologic and molecular abnormalities that are consistent with inflammation are increased in the retinas or vitreous humour of diabetic animals and patients [20, 21]. In particular, this diabetes-induced inflammation is grossly apparent in the retina based on the levels of proinflammatory cytokines, such as tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), VEGF, IL-8, and IL-6 [22–24]. In light of the evidence for the role of IL-1 $\beta$  in inflammation in DR, we hypothesized a role for the NOD-like receptor family, pyrin domain-containing 3 (NLRP3) inflammasome in DR pathogenesis. The NLRP3 inflammasome is a multiprotein complex composed of the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC; also known as PYCARD), the effector cysteine protease 1 (caspase 1), and NLRP3. The NLRP3 inflammasome induces inflammation by mediating the catalytic activation of caspase-1, followed by the cleavage and release of the proinflammatory cytokines IL-1 $\beta$  and IL-18 [25, 26]. Consequently, we can infer that the NLRP3/interleukin-1 $\beta$  pathway plays a critical role in the development and progression of DR.

Vitamin D<sub>3</sub> is essential for a vast number of physiologic processes. In particular, vitamin D<sub>3</sub> may have a suppressive role in the pathogenesis of inflammation and angiogenesis, which are features of DR. Therefore, we hypothesized that vitamin D<sub>3</sub> protects against the development of DR via its anti-inflammatory and antiangiogenic effects.

## 2. Materials and Methods

All experiments in this study complied with the requirements of the Association for Research in Vision and Ophthalmology statement with regard to the “Use of Animals in Ophthalmic and Vision Research.” All chemicals were of reagent grade and were purchased from Sigma Chemicals (St. Louis, MO, USA) unless stated otherwise.

**2.1. Animals.** Eight-week-old male Sprague-Dawley rats weighing 180–200 g (Shanghai Laboratory Animal Center,

Chinese Academy of Sciences) were randomly assigned to receive either 60 mg/kg streptozotocin (STZ) intraperitoneally or citrate buffer alone. Rats were categorized as diabetic when their blood glucose exceeded 16.7 mmol/L at 48 h after STZ administration. Control animals were age matched and given an equal volume of sodium citrate alone instead of STZ. All rats were randomly divided into four groups ( $n = 10$  each):

- (i) Normal control group (NC): SD rats given sodium citrate and administered intramuscular injections of tea oil
- (ii) Normal control group + vitamin D<sub>3</sub> (NC + VD): SD rats administered sodium citrate and intramuscular injections of calcitriol (General, Shanghai, China) at a dose of 233.3 U/kg body weight/week dissolved in tea oil for 6 months, starting 4 weeks after diabetes induction
- (iii) Type 1 diabetes mellitus group (DM): STZ-induced SD diabetic rats administered intramuscular injections of tea oil
- (iv) Type 1 diabetes mellitus group + vitamin D<sub>3</sub> (DM + VD): STZ-induced SD diabetic rats administered intramuscular injections of calcitriol at a dose of 233.3 U/kg body weight/week dissolved in tea oil for 6 months, starting 4 weeks after the induction of diabetes

Different doses (116.7–466.7 U/kg body weight/week) of vitamin D<sub>3</sub> were tested in a preliminary experiment. The dose of 233.3 U/kg body weight/week was the minimum effective dose.

**2.2. Cell Culture and Treatment.** Human retinal microvascular endothelial cells (HRMECs) (Cat. number ACBRI 181, Kirkland, WA, USA) were purchased from Cell Systems with CSC complete medium (glucose concentration of 5 mmol/L), and cells at passage 3–7 were used in the following experiment. Confluent cells were switched to serum-free medium for 24 h before treatment. The cells were exposed to normal glucose (5 mmol/L), normal glucose plus vitamin D<sub>3</sub> (50 nM, D1530 Sigma, high glucose (30 mmol/L), high glucose plus vitamin D<sub>3</sub> (50 nM), high glucose after transfection with an interference plasmid targeting NLRP3, normal glucose after transfection with an overexpression plasmid expressing NLRP3, and high glucose plus the reactive oxygen species (ROS) scavenger N-acetylcysteine (NAC; 10 mmol/L). Each group was incubated for 72 h. In addition, we also added mannitol to the normal glucose medium for osmotic compensation.

**2.3. Plasmid DNA and Transfections.** Transient transfection was carried out using Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen, Grand Island, NY, USA). Briefly, HRMECs were seeded at a density of 10<sup>6</sup>/60 mm plate overnight and then cultured in serum-free culture medium containing 4  $\mu$ g pDNA and 10  $\mu$ L Lipofectamine 2000 for 6 h. Transfected cells were then switched to

normal medium for 12 h before subsequent experiments. The target short hairpin (sh) RNA sequences were 5'-GG AGAGACCTTTATGAGAAAG-3' (Dharmacon) for NLRP3 knockdown. The plasmids encoding full-length NLRP3 were provided by Dr. Haibin Chen (Department of Endocrinology and Metabolism, the Sixth People's Hospital of Shanghai Affiliated to Shanghai Jiao Tong University, Shanghai, China). Both scrambled shRNA control (for knockdown) and empty vector (for overexpression) were used for appropriate controls (Figure 1(c)).

**2.4. Histopathological Evaluation.** In the article of Li et al. [27], authors demonstrated that rosiglitazone attenuated diabetes-induced rat retinal structural abnormality by histopathological evaluation. We followed the methods of Li et al. [27]. Histopathological evaluation was examined by a light microscope (Leica, Heidelberg, Germany). Coloured micrographs were obtained at  $\times 400$  magnification. Measurements ( $\times 400$ ) were made 0.5 mm dorsal and 0.5 mm ventral to the optic disc. The thicknesses of the total retina (between the inner limiting membrane and pigment epithelium), inner plexiform layer (IPL), and inner nuclear layer (INL) and the combined thickness of the outer plexiform and outer nuclear layers (pooled as the outer retinal layers, ORL) were measured. The number of cells in the ganglion cell layer (GCL) was calculated based on the linear cell density (cells per  $100 \mu\text{m}$ ). Three measurements were made at adjacent locations in each hemisphere, and the nine measurements were averaged. The mean of the three eyes was recorded as the representative value for each group. The results of the three groups were subjected to statistical analysis.

**2.5. Apoptosis of Retinal Cells.** The Dead End Fluorometric TUNEL system (Roche TUNEL kit; Roche Applied Science, Burgess Hill, UK) was used as described by the manufacturer. Briefly, paraffin sections were prepared as described by the manufacturer and subjected to TUNEL analysis after incubation with proteinase K; Hoechst counterstaining was then performed. Fluorescence images were acquired using a fluorescence microscope (Olympus, Tokyo, Japan), and images were obtained using Zeiss software version 4.6. Apoptotic cells within the retina fluoresced red.

**2.6. Measurement of Retinal Vascular Permeability.** Retinal vascular permeability was analysed by measuring Evans blue-albumin complex leakage from retinal vessels, as described previously [28]. Briefly, Evans blue dye was dissolved in normal saline (45 mg/mL). Then, under deep anaesthesia, the dye (45 mg/kg) was injected into the jugular vein of each rat. Blood ( $200 \mu\text{L}$ ) was withdrawn from the iliac artery 2 min after Evans blue injection and then every 30 min for up to 120 min. After the dye had circulated for 120 min, the chest cavity was opened, and the left ventricle was cannulated. Each rat was perfused with 0.05 M citrate buffer, pH 3.5 ( $37^\circ\text{C}$ ), for 2 min at 66 mL/min to clear the dye. Immediately after perfusion, the eyes were enucleated, and the retinas were carefully dissected under an operating microscope. Evans blue in the retina and blood samples was detected as described previously [29]. Retinal vascular permeability was

calculated using the following equation, and the results were expressed as  $\mu\text{L}$  plasma/g retina dry weight/h: retinal vascular permeability = Evans blue ( $\mu\text{g}$ )/retina dry weight (g)/time-averaged Evans blue concentration ( $\mu\text{g}$ )/plasma ( $\mu\text{L}$ )  $\times$  circulation time (h).

**2.7. Assessment of HRMEC Apoptosis Using Propidium Iodide (PI)/Hoechst.** To assess cellular apoptosis, HRMECs were stained with Hoechst and PI dyes. Blue fluorescent Hoechst 33342 binds to chromatin and stains the condensed chromatin of apoptotic cells more brightly than that of normal cells, whereas red fluorescent PI permeates late-stage apoptotic and dead cells. HRMECs were stained with  $10 \mu\text{g}/\text{mL}$  PI and  $10 \mu\text{g}/\text{mL}$  Hoechst for 15 min at  $37^\circ\text{C}$  in the dark and then visualized and photographed using a fluorescence microscope (Olympus, Tokyo, Japan) to allow the number of apoptotic cells among 500 total cells to be counted. The percentage of red (apoptotic; R) cells versus bright blue (viable; BB) cells was determined as follows: % apoptotic cells =  $(R + BB)/500$ .

**2.8. Measurement of ROS Production.** Intracellular ROS levels were determined using the oxidation-sensitive fluorescent probe 2',7'-dichlorofluorescein diacetate (H2DCF-DA). Intracellular ROS oxidize this probe into the highly fluorescent compound DCF. Cells were incubated with  $10 \mu\text{M}$  H2DCF-DA in medium at  $37^\circ\text{C}$  for 30 min, and images were acquired using a fluorescence microscope and quantified using ImageJ (National Institutes of Health, Bethesda, MD, USA).

**2.9. Western Blotting.** Total protein samples were separated using 10–12% SDS-polyacrylamide gels and then incubated with the following primary antibodies: NLRP3 (goat polyclonal; Santa Cruz Biotechnology), apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC, rabbit monoclonal; Adipogen International Inc., San Diego, CA, USA), caspase-1 (rabbit polyclonal; Santa Cruz Biotechnology), IL-1 $\beta$  (rabbit polyclonal; Abcam, Cambridge, UK), TXNIP (goat monoclonal; MBL International), vascular endothelial growth factor (VEGF, goat polyclonal; Santa Cruz Biotechnology), BAX (Cell Signaling Technology, Beverly, MA, USA), Bcl-2 (Cell Signaling Technology), and  $\beta$ -tubulin (rabbit polyclonal; Bioworld Technology Inc., St. Louis Park, MN, USA). The blots were washed with Tris-buffered saline containing Tween 20, and signals were developed using enhanced chemiluminescence (Western Chemiluminescent HRP Substrate; EMD Millipore, Billerica, MA, USA).

**2.10. Statistical Analysis.** Results are expressed as mean  $\pm$  standard deviation (SD). Group means were compared by one-way analysis of variance using the GraphPad Prism software (ver. 4.0; GraphPad, San Diego, CA) and the SPSS software (ver. 17.0 for Windows; SPSS Inc., Chicago, IL). In all comparisons, a value of  $P < 0.05$  was considered to indicate a statistical significance.

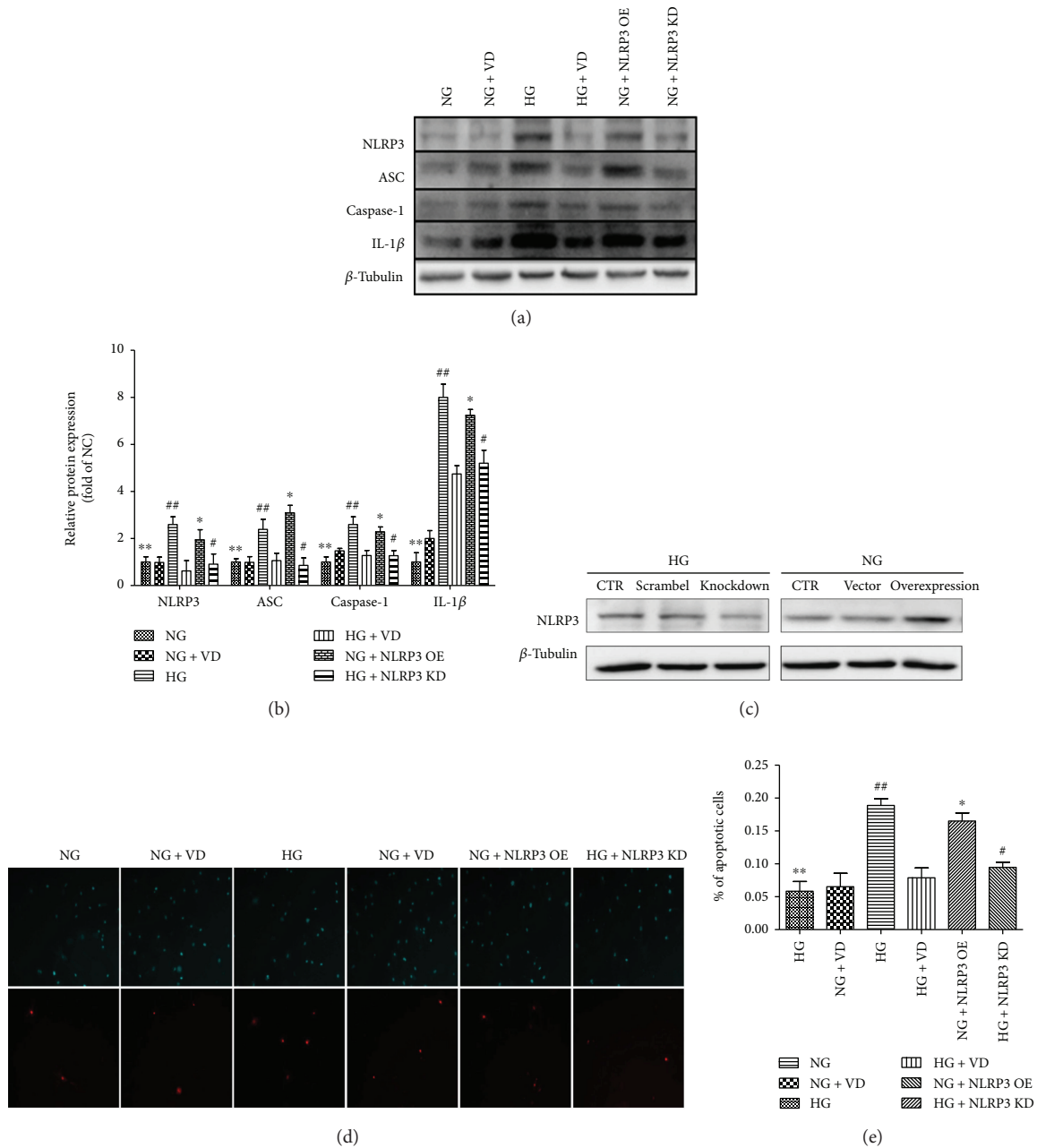


FIGURE 1: Vitamin D<sub>3</sub> inhibits the high-glucose-induced death of HRMECs by inhibiting expression of proteins in the NLRP3 inflammasome pathway. (a) Western blot analysis of proteins in the NLRP3 inflammasome pathway in the NG, NG + VD, HG, HG + VD, and NG + NLRP3-overexpressing and HG + NLRP3-knockdown groups. (b) Quantitative analysis of the bands. (c) Transfection of HRMECs with plasmid DNA (CTR: control group; scramble: scrambled shRNA control group; vector: empty vector control group). (d) Co-staining of Hoechst33238 and propidium iodide was performed to assess apoptosis of HRMECs. (e) Quantification of apoptotic cells. The results are expressed as the mean percentage of apoptotic cells  $\pm$  SD.  $n = 6$  (\*\*: NG versus HG,  $P < 0.05$ ; ##: HG versus HG + VD,  $P < 0.05$ ; \*: NG + NLRP3 Op versus NG,  $P < 0.05$ ; #: HG + NLRP3 KD versus HG,  $P < 0.05$ ).

### 3. Results

**3.1. Vitamin D<sub>3</sub> Inhibits the Decrease in GCL Cell Numbers and Retinal Thickness in Diabetic Rats.** The thicknesses of the total retina, IPL, INL, and ORL and the density of GCL cells in the diabetes mellitus group were significantly lower than those in the NC group ( $P < 0.05$ , Table 1). The cells in each layer of retinal tissue were shaped more

abnormally, arranged in a more disorderly fashion and of a lower density (cells per 100  $\mu$ m) in the DM group than the NC group. However, the retinal structure and cell density of diabetic rats were similar to those in the NC group after treatment with vitamin D<sub>3</sub> (Figure 2(a)). Likewise, measurements of retinal thicknesses and GCL cell numbers showed a significant increase in the DM + VD group compared with the DM group, which confirmed

TABLE 1: Thicknesses of the retinal layers and ganglion cell layer (GCL) and cell counts at month 6.

	Total retina	IPL	Thickness ( $\mu\text{m}$ )		GCL cell numbers (per 100 $\mu\text{m}$ )
			INL	ORL	
NC ( $n = 10$ )	243.1 $\pm$ 3.2	74.3 $\pm$ 2.0	39.2 $\pm$ 2.1	73 $\pm$ 3.5	13.0 $\pm$ 1.5
NC + VD ( $n = 10$ )	245.5 $\pm$ 4.1	79.6 $\pm$ 2.8	41.2 $\pm$ 2.7	76 $\pm$ 2.9	13.0 $\pm$ 2.1
DM ( $n = 10$ )	176.2 $\pm$ 3.7*	54.9 $\pm$ 3.4*	37.3 $\pm$ 3.2*	65 $\pm$ 3.5*	8.3 $\pm$ 0.8*
DM + VD ( $n = 10$ )	229.8 $\pm$ 2.7 <sup>#</sup>	68.7 $\pm$ 3.2 <sup>#</sup>	38.2 $\pm$ 3.1 <sup>#</sup>	69 $\pm$ 3.0 <sup>#</sup>	11.3 $\pm$ 1.3 <sup>#</sup>

NC: normal control group; NC + VD: normal control group + vitamin D<sub>3</sub>; DM: diabetes mellitus group; DM + VD: diabetes mellitus group + vitamin D<sub>3</sub>; IPL: inner plexiform layer; INL: inner nuclear layer; ORL: outer retinal layers; GCL: ganglion cell layer. Data represent mean  $\pm$  SD ( $n = 10$ ). \*NC versus DM,  $P < 0.05$ ; <sup>#</sup>DM + VD versus DM,  $P < 0.05$ . Data are expressed as the mean  $\pm$  standard deviation.

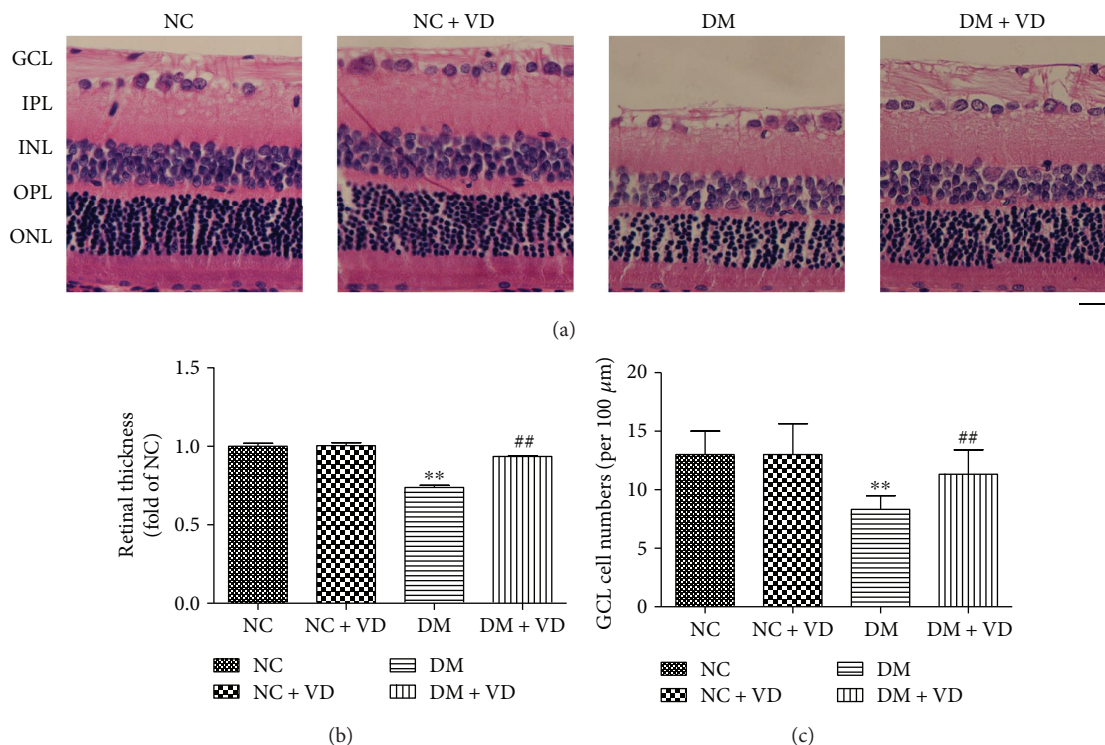


FIGURE 2: (a) Representative images of retinas at month 6. (b-c) The retinal thickness and cell numbers in the GCL (cells per 100  $\mu\text{m}$ ). At month 6, the GCL and IPL were obvious and well organized in the NC group. The retinal thickness in diabetic rats was reduced and the number of GCL cells decreased (DM), whereas in diabetic rats treated with vitamin D<sub>3</sub>, the retinas were more normal in structure and thicker than those in diabetic rats (NC: normal control group; NC + VD: normal control group + vitamin D<sub>3</sub>; DM: diabetes mellitus group; IPL: inner plexiform layer; GCL: ganglion cell layer). Data represent mean  $\pm$  SD ( $n = 10$ ). \*\*DM versus NC,  $P < 0.05$ ; ##DM + VD versus DM,  $P < 0.05$ . Data are expressed as the mean  $\pm$  standard deviation. Scale bar, 30  $\mu\text{m}$ .

the protective effect of vitamin D<sub>3</sub> on the decrease in GCL cell numbers and retinal thickness in diabetic rats (Table 1, Figures 2(b) and 2(c)).

**3.2. Vitamin D<sub>3</sub> Inhibits Retinal Cell Apoptosis in Diabetic Rats by Decreasing the Bax/Bcl-2 Ratio.** To examine retinal cell death, TUNEL staining was performed at month 6 (Figure 3(a)). In the NC and NC + VD groups, the retinal cell nuclei were negative for TUNEL staining, showing no red fluorescence. There were abundant red fluorescent retinal cell nuclei, indicating TUNEL-positive cells, within the inner nuclear, outer nuclear, and ganglion cells in the diabetic rats. In contrast, in the DM + VD group, only sparse red fluorescent nuclei were found in rat retinas by TUNEL staining.

The decreased apoptosis in diabetic rats following vitamin D<sub>3</sub> treatment could be due to changes in Bcl-2 family proteins, which are well-known inducers and integrators of survival and death signals. Therefore, we next determined whether hyperglycaemia affected the levels of the proapoptotic molecule Bax and the antiapoptotic molecule Bcl-2 in diabetic rats using immunoblotting. The Bax/Bcl-2 ratio was upregulated almost eightfold in retinal extracts from diabetic rats and was downregulated by 93.5% after treatment with vitamin D<sub>3</sub> in diabetic rats' retinal extracts.

**3.3. Vitamin D<sub>3</sub> Attenuates the Increase in Blood-Retinal Barrier (BRB) Breakdown in Diabetic Rats by Inhibiting VEGF Expression and the TXNIP/NLRP3 Pathway.**

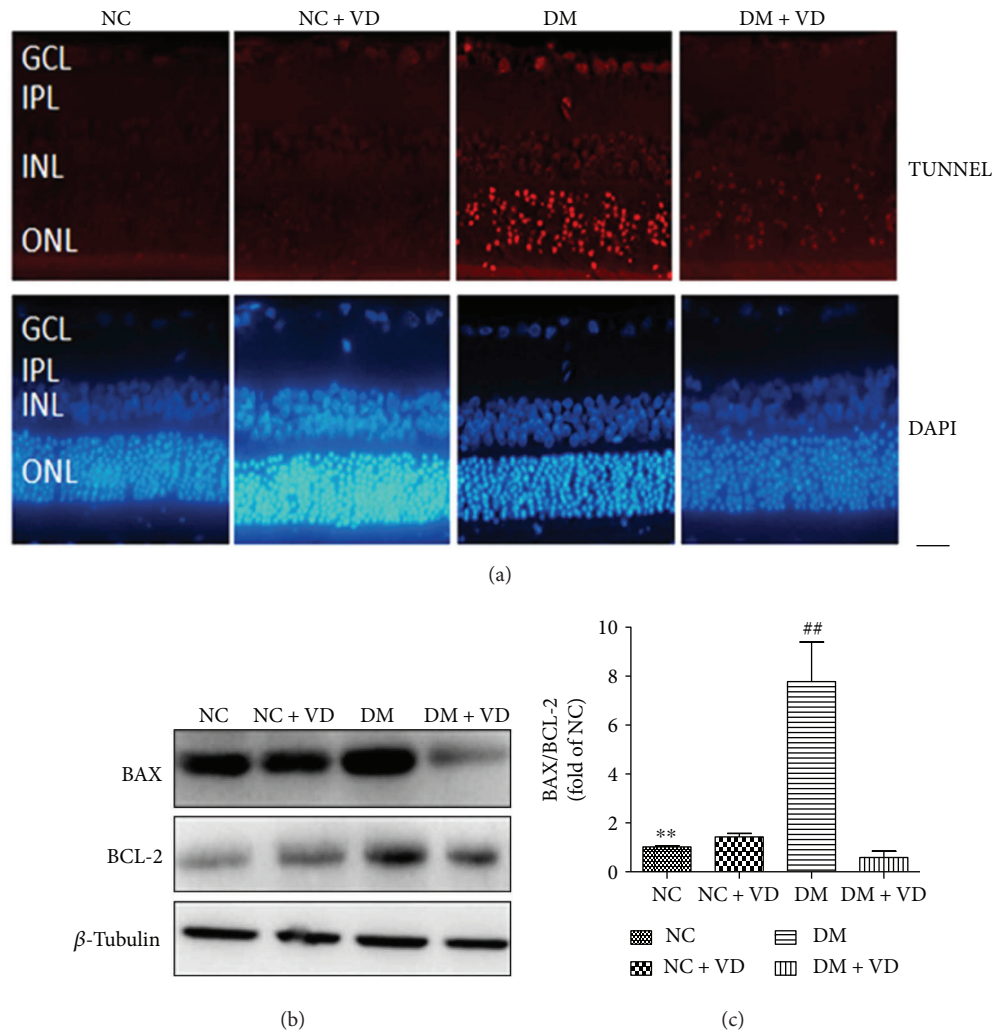


FIGURE 3: Protective effect of vitamin D<sub>3</sub> against apoptosis of retinal cells at month 6. (a) No TUNEL-positive cells were seen in the NC or NC + VD groups; abundant red fluorescent nuclei were observed in the DM group. Sparse red fluorescent nuclei were found in the DM + VD group. GCL: ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer. (b) Western blot of retinal extracts using anti-Bcl-2 and anti-Bax antibodies;  $\beta$ -tubulin was used as the loading control. (c) Bax/Bcl-2 ratios (data represent mean  $\pm$  SD ( $n = 10$ )) \*\*: NC versus DM,  $P < 0.05$ , #: DM versus DM + VD,  $P < 0.05$ . NC: normal control group; NC + VD: normal control group + vitamin D<sub>3</sub>; DM: diabetes mellitus group; DM + VD: diabetes mellitus group + vitamin D<sub>3</sub>. Scale bar, 30  $\mu$ m.

Retinal vascular permeability was quantified by detecting retinal Evans blue leakage. As shown in Figure 4(a), BRB permeability was significantly increased in diabetic rat retinas (DM:  $25 \pm 3$  L plasma/g retina dry weight/h) relative to that in control animals (NC:  $9 \pm 1$  L plasma/g retina dry weight/h;  $P < 0.05$  versus DM). In diabetic rats treated with vitamin D<sub>3</sub>, there was a significant decrease in BRB permeability (DM + VD:  $14 \pm 1$  L plasma/g retina dry weight/h;  $P < 0.05$  versus DM) compared with diabetic animals. Furthermore, VEGF expression and the TXNIP/NLRP3 pathway in rat retinas showed similar trends (Figures 4(c) and 4(e)). The diabetes-induced upregulation of VEGF and proteins in the TXNIP/NLRP3 pathway was prevented by vitamin D<sub>3</sub> treatment.

**3.4. Vitamin D<sub>3</sub> Inhibits Hyperglycaemia-Induced Elevation of TXNIP by Reducing ROS Production in HRMECs.** To assess

the mechanism underlying the inhibitory effect of vitamin D<sub>3</sub> on the TXNIP/NLRP3 pathway, an *in vitro* assay was performed. HRMECs were incubated in low- or high-glucose medium together with 50 nM vitamin D<sub>3</sub> or 10 mM NAC for 48 h. As shown in Figures 5(a)–5(c), high glucose enhanced ROS generation and increased expression of TXNIP ( $P < 0.05$ ), whereas these changes were significantly inhibited by 50 nM vitamin D<sub>3</sub>, which was comparable to the effect induced by treatment with NAC, an ROS scavenger ( $P < 0.05$ ). The cells were treated with mannitol to eliminate the influence of osmolarity; the results showed that mannitol had no effect (data not shown).

**3.5. Vitamin D<sub>3</sub> Inhibits High-Glucose-Induced Death of HRMECs by Inhibiting the Expression of the NLRP3 Inflammasome Pathway.** To confirm the mechanism underlying the effect of vitamin D<sub>3</sub> on HRMEC apoptosis, we

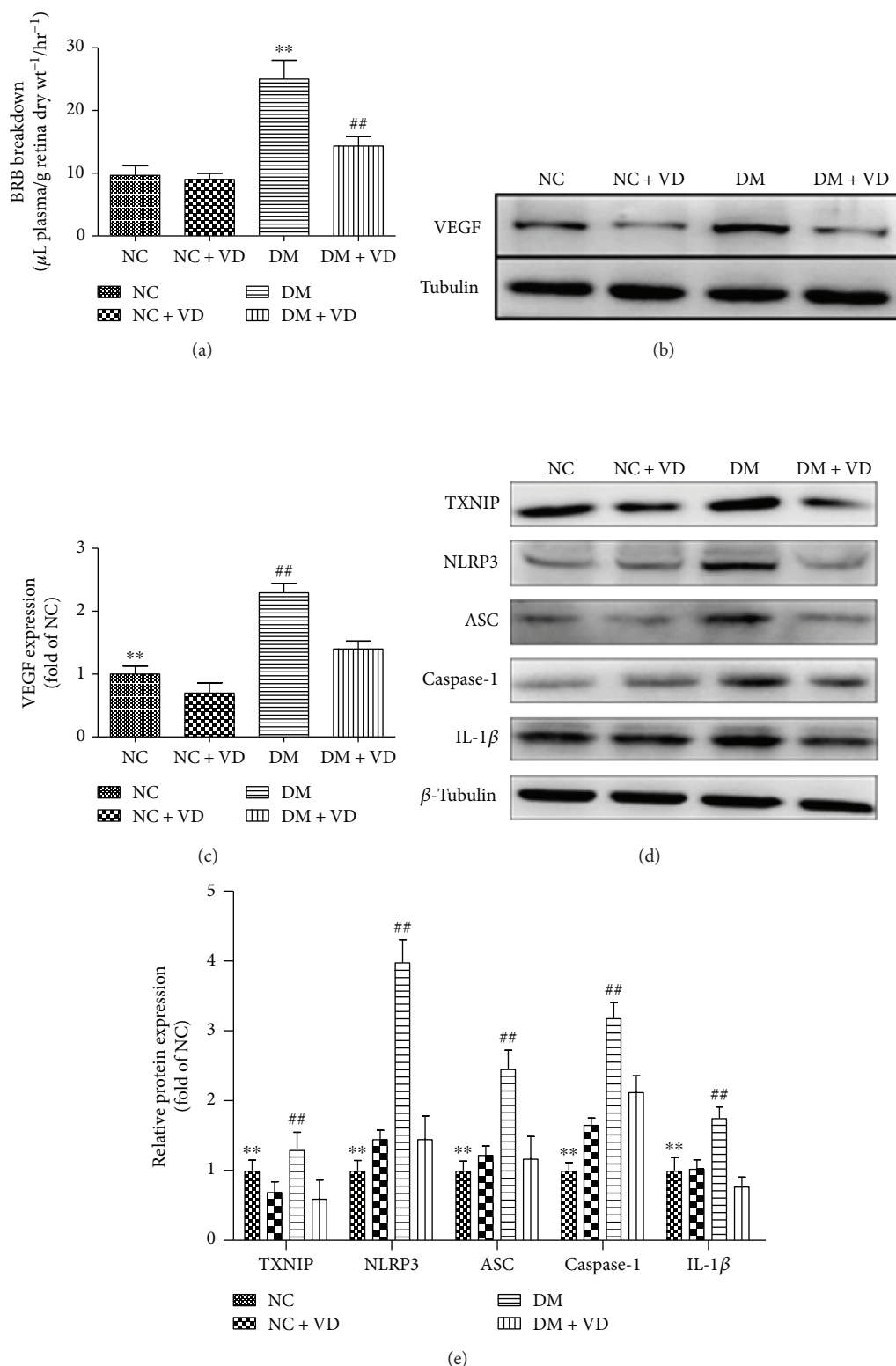


FIGURE 4: Vitamin D<sub>3</sub> attenuates the elevated BRB breakdown in diabetic rats by inhibiting VEGF expression and the TXNIP/NLRP3 pathway. (a) Retinal vascular permeability was quantified by detecting the retinal Evans blue leakage (data are the means  $\pm$  SD,  $n = 10$  \*\*: DM versus NC,  $P < 0.05$  and #: DM + VD versus DM,  $P < 0.05$ ). (b, c) Retinal VEGF protein expression was inhibited by vitamin D<sub>3</sub> (data are the means  $\pm$  SD,  $n = 10$  \*\*: DM versus NC,  $P < 0.05$  and #: DM + VD versus DM,  $P < 0.05$ ). (d, e) Quantitative Western blot analyses of TXNIP and the NLRP3 inflammasome in whole retinal extracts. Expression was normalized to that of  $\beta$ -tubulin. Data are the means  $\pm$  SD,  $n = 10$  (\*\*: NC versus DM,  $P < 0.05$ ; #: DM + VD versus DM,  $P < 0.05$ ).

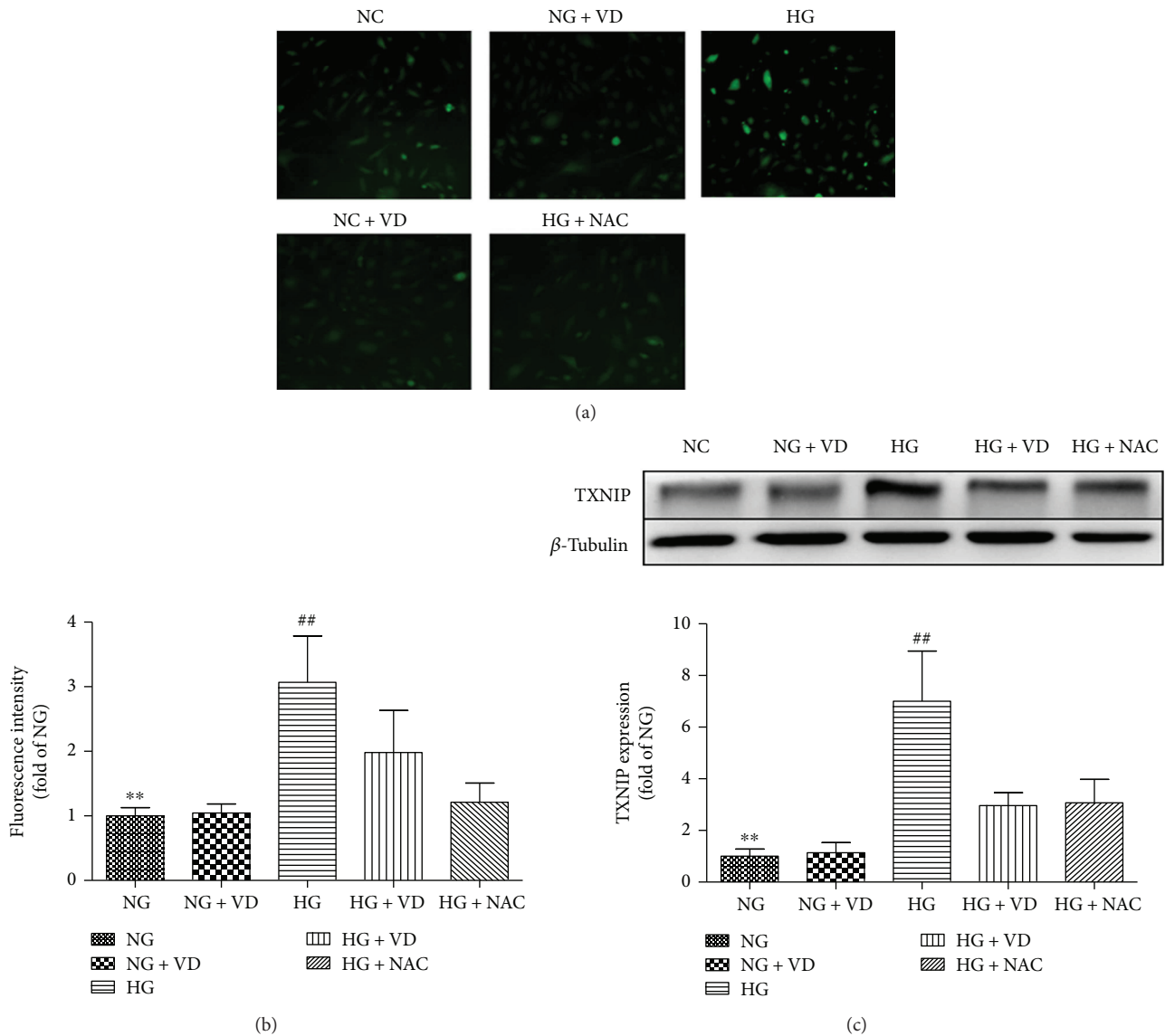


FIGURE 5: Vitamin D<sub>3</sub> inhibits glucose-induced ROS production in HRMECs. (a) To measure ROS production, cells were labelled with H<sub>2</sub>DCF-DA. The figures show representative data from three independent experiments. (b) Quantitative analysis of the measured fluorescence intensity was performed. Values are the means  $\pm$  SD of three independent experiments ( $n = 6$ , \*\*: HG versus NG,  $P < 0.05$ , #: HG + VD and HG + NAC versus HG,  $P < 0.05$ ). (c) Western blot and quantitative analyses of TXNIP from whole retinal extracts. Expression was normalized to that of  $\beta$ -tubulin. Data are the means  $\pm$  SD,  $n = 6$  (\*\*: HG versus NG,  $P < 0.05$ , #: HG + VD and HG + NAC versus HG,  $P < 0.05$ ).

performed Western blotting and an apoptosis assay (Figures 1(a) and 1(d)). HRMECs were treated with vitamin D<sub>3</sub> or plasmid DNA in the presence of various glucose concentrations. When HRMECs were incubated in high-glucose medium for 48 h, the levels of NLRP3 inflammasome pathway proteins increased dramatically, and the rate of apoptosis increased by 4.2-fold ( $P < 0.05$ ). In contrast, the presence of vitamin D<sub>3</sub> in high-glucose medium prevented the increase in the levels of NLRP3 inflammasome pathway proteins and decreased apoptosis by 59.7% ( $P < 0.05$ ). Furthermore, we used plasmid DNA to transfect HRMECs, of which the transfection effects were significant (Figure 1(c)). Consistently, in HRMECs, the levels of NLRP3 inflammasome pathway proteins increased by overexpressing NLRP3

but decreased by knocking down NLRP3. Meanwhile, the rate of apoptosis was regulated by NLRP3 expression. More specifically, the apoptosis rate was significantly reduced by NLRP3 silencing and increased by NLRP3 overexpressing compared with the nontransfected group.

#### 4. Discussion

During the past decade, theories on the functions of vitamin D<sub>3</sub> have been proposed. In addition to its well-established role in calcium and bone homeostasis, vitamin D<sub>3</sub> has been shown to exert autocrine or paracrine immunomodulatory effects [30]. Much epidemiological research has shown that vitamin D<sub>3</sub> deficiency is associated with



a large number of autoimmune and inflammatory diseases, such as rheumatoid arthritis, lupus, inflammatory bowel disease, transplant rejection, cardiovascular disease, infections, and diabetes [6]. Thus, an understanding of the underlying anti-inflammatory mechanisms of vitamin D<sub>3</sub> may extend its clinical applications.

Recently, the relationship between vitamin D<sub>3</sub> deficiency and DR has become a focus of study [8, 9]. Our study found for the first time that vitamin D<sub>3</sub> has a protective effect on DR. Vitamin D<sub>3</sub> markedly reduced retinal injury associated with diabetes, as demonstrated by reduced histological damage and permeability recovery and apoptosis of retinal cells. We also confirmed that the TXNIP/NLRP3 pathway was activated due to increased ROS production induced by high glucose. Vitamin D<sub>3</sub> exerted its protective effect by decreasing the level of ROS production, thus downregulating TXNIP expression and blocking the activation of NLRP3. Eventually, this could lead to the downregulation of IL-1 $\beta$ , which might mitigate retinal inflammation and protect the retina against hyperglycaemia-induced dysfunction.

In an animal study, we found that NLRP3 activation was significantly correlated with damage to retinal cells and vascular permeability, whereas vitamin D<sub>3</sub> downregulated NLRP3, which subsequently reduced the rate of apoptosis and reduced vascular permeability in the retinas of diabetic rats. Furthermore, we found that the Bax/Bcl-2 protein ratio decreased after high-glucose-treated HRMECs were treated with vitamin D<sub>3</sub>. The ratio of proapoptotic Bax to antiapoptotic Bcl-2 in the retina reflects the level of retinal cell apoptosis in diabetic rats [31]. A previous study found that IL-1 $\beta$  induced retinal mitochondrial dysfunction, mitochondrial DNA damage, and cell apoptosis [32]. Bax is a marker of mitochondrial stress associated with vascular diabetes complications [33]. Hence, vitamin D<sub>3</sub> likely reduced the expression of proapoptotic proteins by inhibiting NLRP3-mediated IL-1 $\beta$  secretion. Undoubtedly, VEGF plays a key role in retinal vascular permeability. It was reported that TXNIP increased the expression of VEGF in the diabetic rat retina [34]. In our study, TXNIP exhibited the same trend as VEGF, the levels of which were increased under high-glucose conditions and reversed by vitamin D<sub>3</sub> treatment. These findings indicate that the protective effect of vitamin D<sub>3</sub> on DR is associated with inhibition of the TXNIP/NLRP3 pathway, which may serve as an effective marker of the progression, prevention, and treatment of DR.

To explore the mechanism underlying the protective effect of vitamin D<sub>3</sub> on DR, we performed experiments in vitro. The elevated levels of NLRP3 inflammasome proteins induced by high glucose resulted in high expression of IL-1 $\beta$ , which led to a cellular inflammatory response and death. Overexpression of NLRP3 in the NG group produced similar findings. We also noticed that vitamin D<sub>3</sub> reversed the high-glucose-induced increase in NLRP3 inflammasome protein levels and apoptosis, an effect that was equivalent to the knockdown of NLRP3. These findings suggest that vitamin D<sub>3</sub> protects HRMECs by inhibiting the increase in NLRP3 inflammasome protein levels and decreasing IL-1 $\beta$  secretion.

In our previous studies, we verified that high glucose induces ROS production [35, 36]. Hyperglycaemia-induced ROS production is thought to be a common upstream event in all four classic pathogenetic pathways of diabetic microangiopathy [37–39]. Additionally, two previous studies confirmed that ROS generation is crucial for NLRP3 activation [40, 41]. All NLRP3 agonists trigger the production of ROS, which leads to activation of the NLRP3 inflammasome via the ROS-sensitive TXNIP protein [42]. TXNIP has been reported to be induced by high glucose, and ROS trigger the dissociation of TXNIP from thioredoxin, which alters the function of TXNIP from a thioredoxin repressor to an NLRP3 inflammasome activator. We speculate that vitamin D<sub>3</sub> inhibits NLRP3 by reducing the expression of TXNIP and ROS production. We determined ROS levels and TXNIP expression in HRMECs incubated in the presence of high glucose and NAC. Consistently, our results showed that vitamin D<sub>3</sub> inhibits the expression of TXNIP by degrading ROS in high-glucose-induced HRMECs.

In conclusion, using in vitro and in vivo studies, we demonstrated that vitamin D<sub>3</sub> can protect the normal retinal structure, alleviate retinal vascular permeability, and inhibit the apoptosis of retinal cells in diabetic rats. We also provided evidence that this protective effect was caused by inhibition of the TXNIP/NLRP3 pathway as a result of reduced ROS production in high-glucose-induced retinal microvascular endothelial cells. Thus, vitamin D<sub>3</sub> possesses considerable potential for the treatment of DR. A long-term prospective study with a large number of samples is needed to verify the clinical effect of vitamin D<sub>3</sub> in DR.

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

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