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Bradykinin alleviates DR retinal endothelial injury by regulating HMGB-1/NF-κB pathway

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Abstract. – OBJECTIVE: Diabetic retinopathy (DR) is one of the most important complications of diabetes (DM) and the leading cause of blindness in adults. Bradykinin (BK) is involved in several pathophysiological processes, such as inflammation, pain, cell proliferation, and tumors. It plays a crucial role in corneal epithelial cells, corneal stromal cells, and fibroblasts. However, the role of BK in DR retinal endothelial injury remains unclear.

PATIENTS AND METHODS: Human retinal microvascular endothelial cells (hRECs) were cultured *in vitro* and randomly divided into 3 groups, control group in which hRECs were cultured in normal glucose concentration, high glucose group in which hRECs were cultured in the presence of high glucose, and BK group in which hRECs were cultured in the presence of high glucose with 1 μM BK. The MTT assay (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) was used to detect cell proliferation. Caspase-3 activity was adopted to detect Caspase-3 activity in hRECs. The colorimetric method was selected to determine lactate dehydrogenase (LDH) activity, superoxide dismutase (SOD) activity, and ROS content. Western blot was used to test HMGB-1/NF-κB and vascular endothelial growth factor (VEGF) expression changes. Enzyme-linked immunosorbent assay (ELISA) was performed to detect the secretion of inflammatory factor tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β).

RESULTS: In the presence of high glucose, hRECs cells proliferation was significantly reduced, Caspase-3 activity was enhanced, LDH and ROS levels were increased, SOD activity was declined with increased expression of HMGB-1, NF-κB, VEGF, as well as secretion of TNF-α and IL-1β compared with control group ($p < 0.05$). BK significantly inhibited the proliferation of hRECs cells, enhanced Caspase-3 activity, decreased the content of LDH and ROS, increased SOD activity, reduced the expressions of HMGB-1 and NF-κB protein, attenuated the expression of VEGF, and restrained the secretion of TNF-α and IL-1β compared with high glucose group ($p < 0.05$).

CONCLUSIONS: BK can inhibit the growth and proliferation of retinal endothelial cells by regulating HMGB-1/NF-κB signaling pathway, attenuating oxidative stress and inflammation, thereby delaying DR development and progress.

Key Words:
Bradykinin, HMGB-1/NF-ÐB, Inflammation, Diabetic retinopathy, Retinal endothelial cells.

Introduction

The severe complications of diabetic mellitus (DM) include diabetic retinopathy (DR), which belongs to the fundus lesion and caused by diabetic microangiopathy[1,2]. The incidence of DR is high in the acquired blind eye disease, which severely threatens the health of DM patients[3,4]. It was found that a high glucose environment can cause nerves, blood vessels, and microcirculation changes in the eye tissue, which leads to DR lesions[5]. DR is a chronic progressive inflammatory disease that causes metabolic dysfunction, which leads to an abnormal structure of the retina. It induces a large amount of leukocyte infiltration, adhesion, retinal edema, and hard exudation in the retinal micro vessels, resulting in the complicated pathophysiological processes of DR[6,7]. Retinal endothelial cells (RECs) play a regulatory role in anti-toxins and inflammatory factors, protecting blood-retinal barriers, and retinal neurotrophic supplement[8,9]. Long-term hyperglycemia stimulation on retinal microvascular endothelial cells can cause apoptosis or dysfunction of cells, and promote DR progression[10]. Inflammation and oxidative stress can cause endothelial cell damage, induce blood-retinal barrier destruction, and increase vascular permeability[11,12].

Bradykinin (BK) belongs to the 9-peptide kinin substance in the kinin system. It is synthesized by the liver and kidney and is widely distributed

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in the salivary glands, kidney, pancreas, and central nervous system[12,13]. BK is a vasoactive substance that has been shown to be capable to protect the heart, reduce the area of myocardial infarction in acute ischemia-reperfusion and delayed myocardial injury, which is induced by ischemia-reperfusion[14]. It was confirmed that BK in the kallikrein-kinin system can participate in several pathophysiological processes, such as inflammation, pain, cell proliferation, and tumors. It plays an important role in the proliferation of corneal epithelial cells, corneal stromal cells, and fibroblasts[15,16]. However, the role of BK in DR retinal endothelial injury has not been elucidated.

Materials and Methods

Main Reagents and Instruments

Human retinal endothelial cells (hRECs) were purchased from Angio-Proteomie. Bradykinin (BK) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM) and penicillin-streptomycin were purchased from Hyclone (South Logan, UT, USA). Dimethyl sulfoxide (DMSO) and MTT powder were purchased from Gibco (Grand Island, NY, USA). Trypsin-EDTA was purchased from Sigma-Aldrich (St. Louis, MO, USA). Polyvinylidene difluoride (PVDF) membrane was purchased from Pall Life Sciences (Port Whashington, NY, USA). Ethylene diamine tetraacetic acid (EDTA) was purchased from Hyclone Corporation. Western blot related chemical reagents were purchased from Beyotime (Shanghai, China). Enhanced chemiluminescence (ECL) reagents were purchased from Amersham Biosciences (Buckinghamshire, UK). Rabbit anti-human HMGB-1 monoclonal antibody, rabbit anti-human NF-κB monoclonal antibody, rabbit anti-human VEGF monoclonal antibody, and goat anti-rabbit horseradish peroxidase (HRP)-labeled IgG secondary antibody were purchased from Cell Signaling Technology (Danvers, MA, USA). TNF-α and IL-1β. Enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D (Minneapolis, MN, USA). Caspase 3 activity detection kit was purchased from Pall Life Sciences. SOD activity kit, LDH activity kit, and ROS content kit were purchased from Wuhan Boster Biotechnology Co., Ltd. The Gene Amp PCR System 2400 DNA Amplifier was purchased from PE Corporation. The Labsystem Version 1.3.1 microplate reader was purchased from Bio-Rad Corporation (Bio-Rad, Hercules, CA, USA). The ABI 7700 Fast Quantitative PCR Reactor was purchased from ABI (Vernon, CA, USA). Other commonly used reagents were purchased from Sangon (Shanghai, China).

Methods

HRECs Cell Culture and Grouping

The hRECs were inoculated in a culture dish at a density of 1×10^6 cells/cm^2. The culture medium was supplemented with nerve cell medium, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5.5 mmol/L glucose. The cells were randomly divided into 3 groups, including the control group in which hRECs were cultured in normal glucose concentration, high glucose group in which hRECs were cultured in 30 mmol/L glucose condition, and BK group in which hRECs were cultured under 30 mmol/L glucose condition and treated with 1 μM BK. This investigation was approved by the Ethic Committee of the Affiliated Hospital of Weifang Medical University.

MTT Assay

The cells in logarithmic phase were seeded in 96-well plate at 3000 cells/well and 20 μl MTT was added and incubated for 4 h. Then, 150 μl dimethyl sulfoxide (DMSO) was added to the plate and incubated for 10 min followed by measuring the absorbance value (A) at 570 nm. The proliferation rate = A in test group/A in control ×100%.

Western Blot

The cells were lysed by radioimmunoprecipitation assay (RIPA) lysis buffer (150 mM NaCL, 1% NP-40, 0.1% SDS, 2 μg/ml Aprotinin, 2 μg/ml Leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1.5 mM EDTA, 1 mM NaVanadate) and quantified by the bicinchoninic acid (BCA) method. The isolated proteins were electrophoresed using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was transferred to PVDF membrane by semi-dry transfer method at 100 mA for 1.5 h. After blocked for 1 h, the membrane was incubated with VEGF, HMGB-1, and NF-κB (1:1000, 1:1000, 1:2000) primary antibodies at 4°C overnight. After incubated with secondary antibody (1:2000) under the dark for 30 min, the membrane was imaged using chemiluminescence reagent for 1 min and analyzed by image processing system software and Quantity one software. The experiment was repeated four times (n=4).

Caspase 3 Activity Detection

Caspase3 activity in each group of cells was examined by the kit according to the kit instructions. The cells were trypsinized and centrifuged at 4°C at 600 g for 5 min. Next, the cells were added with lysate on ice for 15min and centrifuged at 4°C at 20000 g for 5 min. Then, the cells were treated with 2 mM Ac-DEVD-pNA and the optical density (OD) value was detected at 405 nm wavelength.

ELISA

ELISA was used to measure TNF-α and IL-1β contents in the serum. A total of 50 μl diluted standard substance were added to each well to establish a standard curve. Next, the plate was added with 50 μl sample and washed for five times. Then, the plate was incubated in 50 μl conjugate reagent at 37ºC for 30 min. At last, the plate was tested at 450 nm to obtain the OD value. The OD value of the standard substance was used to prepare the linear regression equation, which was adopted to calculate the concentration of samples.

ROS Content Detection

The treated cells were bathed in a 95°C water for 40 min and rinsed with cold water. After centrifuged at 4000 rpm for 10 min, the cells were incubated with 2',7'-dichlorofluorescein diacetate (DCF-DA) at 37°C for 15 min and centrifuged at 10,000 rpm for 15 min. The sediment was resuspended in sterile phosphate-buffered saline (PBS) buffer and incubated at 37°C for 60 min. The level of ROS was measured by a microplate reader.

LDH Activity and SOD Activity Detection

SOD activity was examined according to the kit instruction. The protein was extracted and washed in a 95°C water bath. After 40 min, it was taken out and rinsed with cold water. After centrifuged at 4000 rpm for 10 min, the sample was extracted using an ethanol-chloroform mixture (5:3, v/v volume ratio 5:3) to detect total the SOD activity. LDH activity was determined according to the kit instruction. The tissue was loaded into an Eppendorf (Ep) tube and bathed at 95°C for 40 min. After centrifuged for 10 min, the sample was mixed in a buffer containing 30 mM H_2O_2 phosphate (pH 7.0) and bathed for 10 min to determine the change of enzyme activity.

Statistical Analysis

All data analyses were performed on SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). The measurement data were presented as mean ± standard deviation and compared by one-way ANOVA. $p < 0.05$ was considered as statistical difference.

Results

The Impact of BK on DR Retinal Endothelial Cell Proliferation

The effect of BK on the proliferation of DR retinal endothelial cells was analyzed by MTT assay. HRECs proliferation was significantly inhibited in the high glucose environment compared with control ($p < 0.05$). The administration of BK in a high glucose environment significantly promoted cell proliferation compared with the high glucose group ($p < 0.05$) (Figure 1).

The Influence of BK on Caspase 3 Activity in DR Retinal Endothelial Cells

The activity of Caspase3 in hRECs increased apparently in a high glucose environment compared with control group ($p < 0.05$). The administration of BK in a high glucose environment significantly suppressed Caspase3 activity compared with high glucose group ($p < 0.05$) (Figure 2).

The Effect of BK on LDH and SOD Activities in DR Retinal Endothelial Cells

The effect of BK on oxidative stress in DR retinal endothelial cells was further analyzed. Under a high glucose environment, LDH activi-

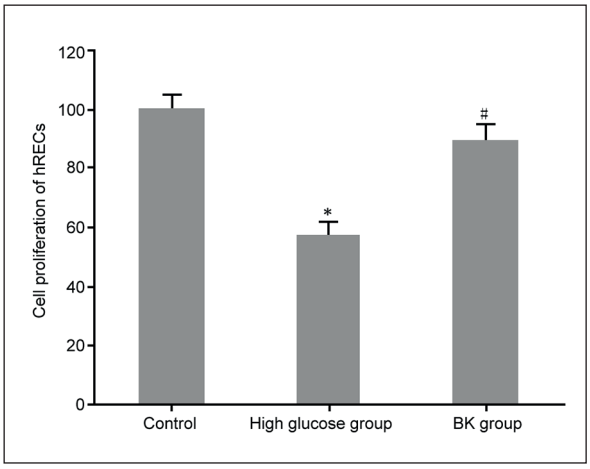

Figure 1. The impact of BK on DR retinal endothelial cell proliferation. *$p < 0.05$, compared with control. #$p < 0.05$, compared with high glucose group.

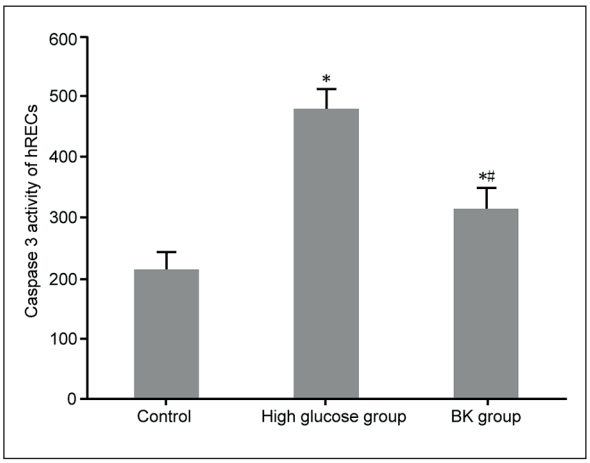

Figure 2. The influence of BK on Caspase 3 activity in DR retinal endothelial cells. *$p < 0.05$, compared with control. # $p < 0.05$, compared with high glucose group.

ty was significantly enhanced and SOD activity was significantly decreased compared with control group ($p < 0.05$). BK administration significantly restrained LDH activity and promoted SOD activity under high glucose environment compared with the high glucose group ($p < 0.05$) (Figure 3).

The Impact of BK on ROS Content in DR Retinal Endothelial Cells

In a high glucose environment, the ROS content of DR retinal endothelial cells was significantly elevated compared with the control group ($p < 0.05$). BK administration markedly declined ROS content in hRECs compared with high glucose group ($p < 0.05$) (Figure 4).

The Influence of BK on Inflammatory Factors Secretion in the Supernatant of DR Retinal Endothelial Cells

The effect of BK on the expression of inflammatory factors TNF-α and IL-1β in the supernatant of DR retinal endothelial cells was analyzed. It was demonstrated that the expressions of TNF-α and IL-1β in the supernatant of DR retinal endothelial cells were significantly increased in high glucose environment compared with the control ($p < 0.05$). BK significantly attenuated the secretion of inflammatory factors TNF-α and IL-1β in hRECs compared with the high glucose group ($p < 0.05$) (Figure 5).

The Effect of BK on VEGF Expression in DR Retinal Endothelial Cells

VEGF expression was significantly enhanced in high glucose environment compared with the control ($p < 0.05$). BK significantly downregulated VEGF expression in hRECs compared with the high glucose group ($p < 0.05$) (Figure 6).

The Impact of BK on HMGB-1/NF-κB Signaling Pathway in DR Retinal Endothelial Cells

HMGB-1 and NF-κB expressions were significantly upregulated in high glucose environment compared with the control ($p < 0.05$). BK signifi-

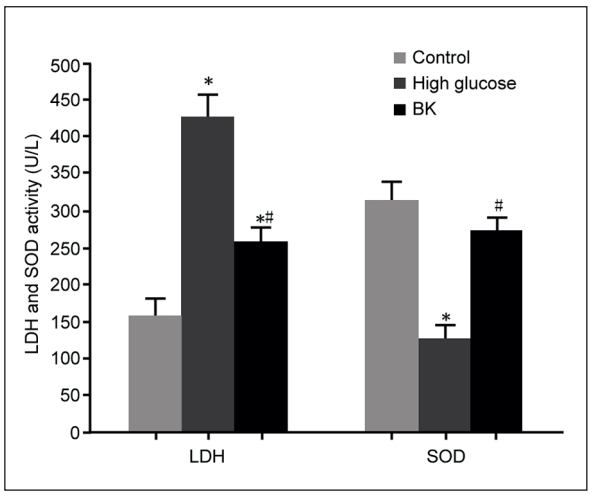

Figure 3. The effect of BK on LDH and SOD activities in DR retinal endothelial cells. *$p < 0.05$, compared with control. # $p < 0.05$, compared with high glucose group.

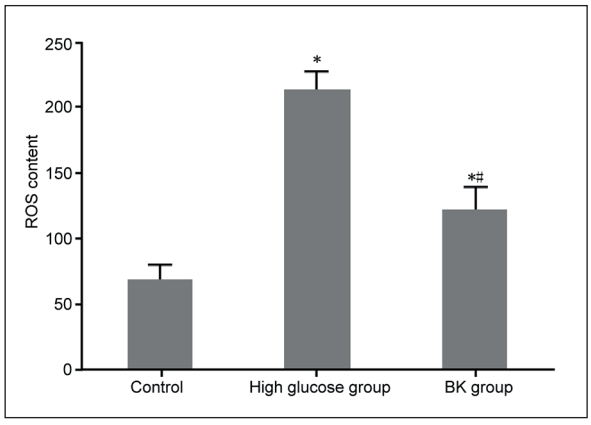

Figure 4. The impact of BK on ROS content in DR retinal endothelial cells. *$p < 0.05$, compared with control. # $p < 0.05$, compared with high glucose group.

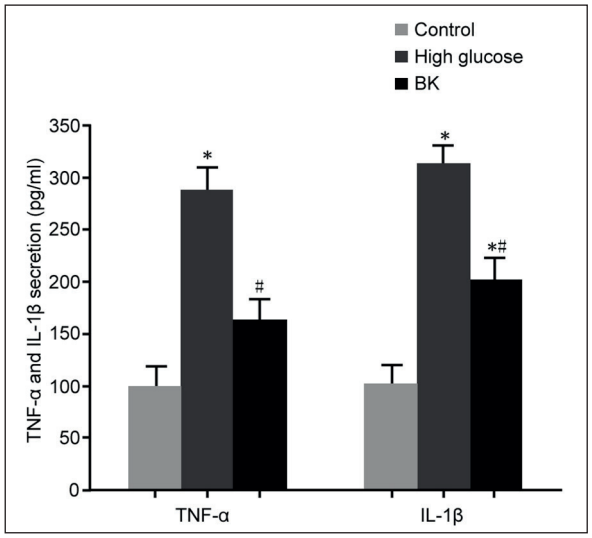

Figure 5. The influence of BK on inflammatory factors secretion in the supernatant of DR retinal endothelial cells. *$p < 0.05$, compared with control. #$p < 0.05$, compared with high glucose group.

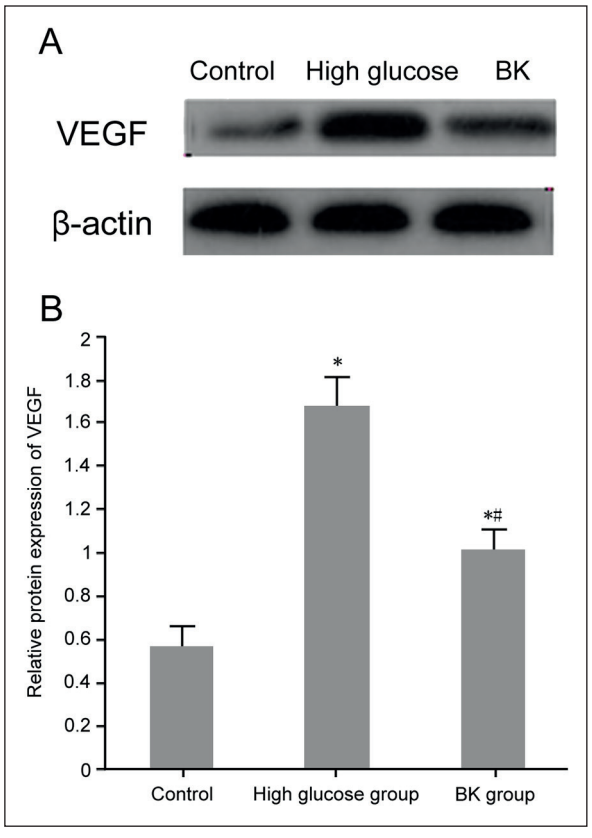

Figure 6. The effect of BK on VEGF expression in DR retinal endothelial cells. **A,** Western blot detection of VEGF protein expression; **B,** VEGF protein expression analysis. *$p < 0.05$, compared with control. #$p < 0.05$, compared with high glucose group.

cantly restrained HMGB-1 and NF-κB expressions in hRECs compared with the high glucose group ($p < 0.05$) (Figure 7).

Discussion

Microvascular disease in diabetic patients mainly occurs in the retina and kidney, which is the main cause of blindness, renal failure, and death[17]. DR is a common microvascular complication of diabetes. The changes of ischemia and hypoxia can cause retinal or optic disc neovascularization, preretinal hemorrhage, and traction retinal detachment, leading to progressive damage of retinal micro vessels. It seriously affects patients' physical and mental health[18,19]. In a hyperglycemic environment, it changes endocrine metabolism, resulting in retinal endothelial cells dysfunction and damage[20]. With

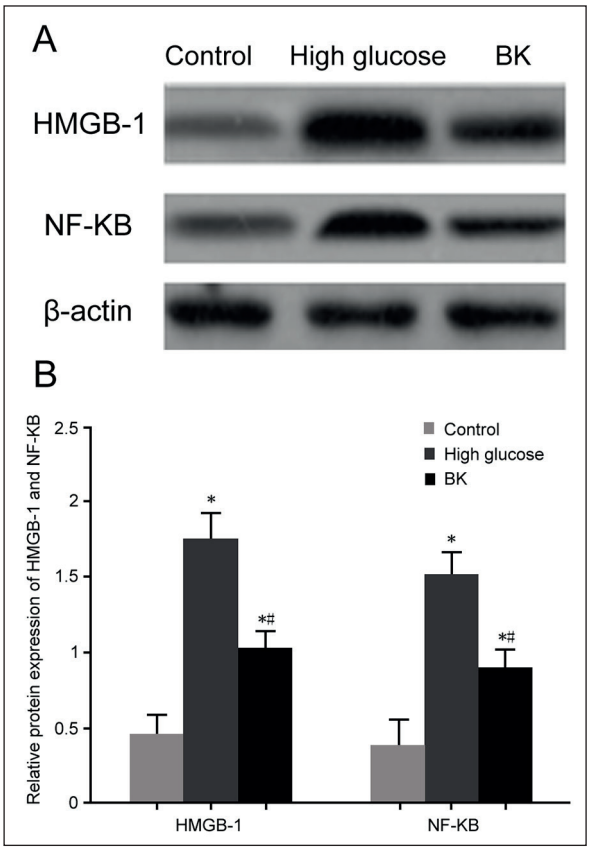

Figure 7. The impact of BK on HMGB-1/NF-κB signaling pathway in DR retinal endothelial cells. **A,** Western blot detection of HMGB-1/NF-κB signaling pathway protein expression; **B,** HMGB-1/NF-κB signaling pathway protein expression analysis. *$p < 0.05$, compared with control. #$p < 0.05$, compared with high glucose group.

the continuous advancement of medical technology, the treatment of DR has gradually become more diversified and individualized. However, the treatment effect of DR is unsatisfactory with poor prognosis[21].

BK plays various roles, including promoting vasodilation, smooth muscle contraction, cell proliferation and repair, and vascular permeability regulation[12,13]. This work found that in high glucose environment, BK promoted hRECs proliferation and decreased cell apoptosis, suggesting that BK might be involved in the repair of RECs in DR. Hyperglycemia causes excessive production of ROS, increase LDH activity, reduces SOD activity, and induces dynamic imbalance of oxidation and antioxidant systems, thereby leading to the release of inflammatory cytokines. Increased expression of adhesion molecules can lead to podocytes apoptosis and damage, resulting in retinal endothelial cells dysfunction and damage, as well as enhanced neovascularization[22,23]. In the further analysis of the mechanism of BK-regulated DR-induced RECs injury, BK can down-regulate VEGF expression, which may be related to competitive binding to VEGF receptors. At the same time, it restrained the secretion of inflammatory factors and regulated oxidative stress. Moreover, it was found that the expression of HMGB-1/NF-KB pathway was significantly increased in DR patients, which could promote angiogenesis and induce inflammation in diabetic retinopathy[24,25]. This study further revealed that BK can suppress the oxidative stress and release of inflammatory factors, downregulate the expression of VEGF, and regulate the growth and proliferation of RECs by inhibiting the HMGB-1/NF-KB signaling pathway. This study preliminarily analyzed the role of BK in DR retinal endothelial cells at the cellular level. Further research is required to explore the role of BK in clinical patients and possible related mechanisms to provide a relevant reference for clinical DR treatment.

Conclusions

We showed that BK could inhibit the growth and proliferation of retinal endothelial cells by regulating the HMGB-1/NF-κB signaling pathway, attenuating oxidative stress and inflammation, thereby delaying DR development and progress.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

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