Ranibizumab prevents Müller cell edema by decreasing VEGF-A in diabetic retinopathy

CURRENT STATUS: UNDER REVIEW

Tianqin Wang
Shanghai Jiao Tong University School of Medicine Affiliated Renji Hospital

Chaoyang Zhang
Shanghai Tenth People's Hospital

Hai Xie
Shanghai Tenth People's Hospital

Qiuxue Yi
Shanghai Jiao Tong University School of Medicine Affiliated Renji Hospital

Dandan Liu
Shanghai Tenth People's Hospital

Qing Peng
Shanghai Tenth People's Hospital

Weiye Li
Drexel University College of Medicine

Haibin Tian
Shanghai Tenth People's Hospital

Lixia Lu
Shanghai Tenth People's Hospital

Jing-Ying Xu
Shanghai Tenth People's Hospital

Guo-Tong Xu
Shanghai Tenth People's Hospital

Lin Liu
Shanghai Jiao Tong University School of Medicine Affiliated Renji Hospital

Jingfa Zhang 13917311571@139.com
Shanghai First People's Hospital

Corresponding Author
Abstract

Background: Diabetic macular edema (DME) is the most common cause of vision loss in patients with diabetic retinopathy. The efficacy of anti-VEGF therapy has been well demonstrated and become the standard of care in the management of DME. The present study is to explore the possible mechanism(s) of ranibizumab in protecting Müller cells from cellular edema in experimental diabetic retinopathy.

Methods: Sprague-Dawley rats were rendered diabetes with intraperitoneal injection of streptozotocin. Intravitreal injection of ranibizumab was performed 8 weeks after diabetes onset. Four weeks later, the rats were killed and the retinas were harvested for examination. rMC-1 cells (rat Müller cell line) were treated with glyoxal for 24 hours, with or without ranibizumab. Cell viability was detected with CCK-8 assay. The expressions of inwardly rectifying K⁺ channel 4.1 (Kir4.1), aquaporin 4 (AQP4), Dystrophin 71 (Dp71), vascular endothelial growth factor A (VEGF-A), glutamine synthetase (GS) and sodium-potassium-ATPase (Na⁺ -K⁺ -ATPase) were examined with Western blot. VEGF-A in the supernatant of cell culture was detected with ELISA. The intracellular potassium and sodium levels were detected with specific indicators.

Results: Compared to the normal control, the protein expressions of Kir4.1, AQP4 and Dp71 were down-regulated significantly in diabetic rat retinas, which were prevented by ranibizumab. The above changes were recapitulated in vitro. As compared with the control, the intracellular potassium level in glyoxal-treated rMC-1 cells was increased, while the intracellular sodium level and Na⁺ -K⁺ -ATPase protein level remained unchanged. However, ranibizumab treatment increased Na⁺ -K⁺ -ATPase protein expression and decreased intracellular sodium, but not potassium level.

Conclusion: Ranibizumab protected Müller cells from intracellular edema through up-regulation of Kir4.1, AQP4, and Dp71 by directly binding VEGF-A. It also increased the
expression of Na + -K + -ATPase, contributing to reduction of the intracellular osmotic pressure.

Background

Diabetes mellitus (DM) is one of the most common chronic diseases with rapidly increased prevalence throughout the world[1]. Diabetic retinopathy (DR), the major microvascular complication of DM, is the leading cause of blindness in working-age people, in which central involved diabetic macular edema (DME) contributes most[2, 3].

Retinal edema, driven by Starling equation, results from the imbalance between fluid entry, fluid exit and retinal hydraulic conductivity, which leads to intraretinal or subretinal fluid accumulation. Under physiological condition, influx and efflux of the ion and water is balanced by the integrity of blood-retinal barrier (BRB) and the normal functions of Müller cells and retinal pigment epithelium (RPE). However, in DME, the increased fluid entry and decreased drainage function results in intracellular and extracellular edema of macula. Among all, the breakdown of inner BRB plays the most important role, caused by junctional complex alternation[4], enhanced transcellular permeability[5], loss of endothelial cells[6], loss of pericytes[7] and vessel abnormalization[8]. Moreover, the dysfunction and death of RPE cells impairs outer BRB[9]. Dysfunction of Müller glia and RPE also contribute the fluid accumulation in the parenchyma of the retina and subretinal space leading to intracellular and extracellular edema[10].

In our clinical study, a strong correlation was found between central subfield thickness (CSFT) and the thickness of inner nuclear layer (INLT) in severer DME (CSFT > 275 µm), suggesting that intracellular edema, particularly Müller glial edema, contributes to DME formation[11]. Müller cells, as specific macroglia in retina, regulate the homeostasis of ion and water mainly through Kir4.1 and AQP4 on the plasma membrane[12-14]. The polarized distribution of Kir4.1 enables the efflux of potassium away from neural
retina[13]. Water, accompanied with potassium and powered by osmotic pressure, is transported through AQP4, a selective water transport protein co-localized with Kir4.1. Both Kir4.1 and AQP4 were anchored by Dp71 on the membrane of Müller cell[15, 16]. It was reported that the swelling of Müller cells is caused by the downregulation or redistribution of Kir4.1, AQP4 and Dp71 in many disease models, such as retinal vein occlusion and ischemia-reperfusion injury[17, 18].

Since VEGF acts as a key mediator in the pathogenesis of DME, anti-VEGF therapy has provided insights into the management of this vision-threatening disease. Nowadays, intravitreal anti-VEGF therapy has become the standard of care in the management of sight-threatening DME. However, whether anti-VEGF reagent, e.g., ranibizumab, could directly affect the expressions or distribution of Kir4.1, AQP4 and Dp71 in diabetic retina still remains unknown. In this study, the potential mechanism of ranibizumab in protecting Müller cell from edema in DR was explored both in vivo and in vitro. The data showed that ranibizumab, through binding VEGF, maintained the expressions of Kir4.1, AQP4 and Dp71 in both diabetic rat retinas and glyoxal-treated Müller cell line. Anti-VEGF agent can also prevent Müller cell from swelling by decreasing the intracellular osmotic pressure.

Methods

Reagents and antibodies

Streptozotocin (STZ, S0130) and glyoxal (50649) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco’s modified Eagle’s medium (DMEM) Low Glucose Medium (SH30021.01B) was purchased from HyClone Laboratories (Logan, UT, USA). Penicillin-streptomycin (15140155) was purchased from Invitrogen (Carlsbad, CA, USA). The primary antibodies against Kir4.1 (APC-035) and AQP4 (AQP-004) were purchased from Alomone Lab (Jerusalem, Israel). Dystrophin (ab7164), GFAP (ab53554) and Na⁺-K⁺-ATPase
(ab76020) antibodies were purchased from Abcam (Cambridge, UK). Glutamine synthetase (GS, NBP2-43646) and VEGF-A (NB100-664) antibodies were purchased from Novus Biological (Littleton, USA). Secondary antibodies, anti-mouse IgG (H+L) (5257S) and anti-rabbit IgG (H+L) (5366S), were purchased from Cell Signaling Technology (Beverley, MA, USA). Protein extraction radioimmunoprecipitation assay (RIPA) buffer (PC101) was purchased from Shanghai Yeasen Biotechnology Co. Ltd. (Shanghai, China). Rat VEGF Quantikine ELISA Kit (RRV00) was purchased from R&D Systems, Inc (Minneapolis, MN, USA). Potassium indicator (P1267MP) and sodium indicator (S1263) were purchased from Thermo Fisher Scientific (Shanghai, China).

**Experimental animals and intravitreal ranibizumab injection**

Male Sprague-Dawley rats weighing 120 to 150 g (Slaccas, Shanghai, China) were used. They were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and The Guides for the Care and Use of Animals (National Research Council and Tongji University). The protocol was approved by the Committee on the Ethics of Animal Experiments of Tongji University (Permit No. TJLAC-017-031). Totally 60 rats were randomly divided into three groups: normal control (N), diabetic rats (D), and diabetic rats treated with intravitreal ranibizumab (D+R). Diabetes was induced by intraperitoneal injection of STZ (60 mg/kg body weight, dissolved in citrate buffer, pH 4.5) and the control rats received an equal volume of citrate buffer. The rats with blood glucose level exceeding 300 mg/dL for 3 consecutive days were considered as diabetes rats and were included in this study. Intravitreal injection was performed in diabetic rats 8 weeks after diabetes onset. Ranibizumab (20 μg/eye, 2μL) was injected intravitreally with a microsyringe (Hamilton, Reno, NV, USA) through a 30-gauge, 0.5-in. needle (BD Biosciences, Franklin Lakes, NJ, USA), which was inserted into the eye 2 mm posterior to the limbus at the temporal side. For normal control and diabetic control, the same volume
(2 μL) of normal saline was injected. Four weeks after the injection, the rats were sacrificed and the eyes were enucleated for the following study.

**Rat Müller cell (rMC-1) culture**

Transformed rat retinal Müller cell line (rMC-1) was kindly supplied by Sarthy (Northwestern University, Chicago, IL, USA). The cells were cultured in low glucose (5.5 mM) DMEM containing 10% fetal bovine serum (Gibco, Shanghai, China) and 1% penicillin/streptomycin at 37°C with 5% CO₂ in a humidified incubator. When the cells reached 80~90% confluence in 10-cm dish, they were divided into three groups, i.e., normal control (N), glyoxal (1 mM)-treated group (G), and glyoxal (1 mM) + ranibizumab (0.125 mg/mL)-treated group (G+R).

**Cell viability assay**

Cell viability of rMC-1 cells was measured using the Cell Counting Kit-8 (CCK-8) assay. Briefly, the rMC-1 cells, incubated with different doses of glyoxal (0.1-5 mM), were seeded on 96-well plates at a density of 10⁴ cells per well treated with or without ranibizumab (0.125 mg/mL) for 1 to 36 hours. The cells were washed with phosphate-buffered saline (PBS), and then incubated with serum-free low glucose DMEM containing 10% of CCK-8 for 3 hours at 37°C. The absorbance was measured at 450 nm by using a microplate spectrophotometer (Tecan, Crailsheim, Germany). The cell viability was expressed as the percentage of the untreated control, which was defined as 100% for each experiment.

**RNA extraction and real-time PCR**

Total RNA was extracted from rMC-1 cells. Reverse transcription was performed and real-time PCR was carried out by using SYBR Green Real-Time PCR master mix (Toybo, Osaka, Japan). The primers were designed using the software Primer Premier Version 5.0 and were ordered from Shanghai DNA Biotechnology Co. Ltd. (Shanghai, China). The primer
information was listed in Table 1.

| Primer name | Sequence | Product size (bp) |
|-------------|----------|-------------------|
| Kir4.1      | Sense    | 5’-TTACAGGCCAGACGACGCA GACA- 3’ |
|             | Antisense| 5’- ACCAGATACCACACCACG CCAA- 3’ |
| AQP4        | Sense    | 5’- GGAAGGCATGAGTGACGG AG- 3’ |
|             | Antisense| 5’- TGCTGAGTCCAAAGCAGA GG- 3’ |
| Dp71        | Sense    | 5’- ATGAGGGAACAGCTCAA GG- 3’ |
|             | Antisense| 5’- TGCAGCTGACAGGCTCAA GA- 3’ |
| GS          | Sense    | 5’- CAGAGACCAACCTGAGGC ACAG- 3’ |
|             | Antisense| 5’- GCTCCACACCGCAGTAA TAGG- 3’ |
| VEGF-A      | Sense    | 5’- GCACATAGGAGAGATGAG CTTCC- 3’ |
|             | Antisense| 5’- CTCCGCTCTGAACAAGGC T- 3’ |
| β-Actin     | Sense    | 5’- GTAAAGACCTCTATGCCA ACA- 3’ |
|             | Antisense| 5’- GGACTCATCGTACTCCTG CT- 3’ |

### Protein extraction and Western blot

The proteins were extracted from the retinas and rMC-1 cells lysed in RIPA buffer and the protein concentrations were determined with Pierce BCA Protein Assay Kit (Thermo Scientific). Equal amounts of protein were resolved on 10% SDS-polyacrylamide gels and transferred electrophoretically onto nitrocellulose membranes (Bio-Rad, Shanghai, China). The membranes were blocked in 5% Tris buffered saline Tween-20 (TBST) buffered bovine serum albumin at room temperature for 30 minutes, and then incubated separately with antibodies against Kir4.1 (1: 500), AQP4 (1: 1,000), Dystrophin 71 (1: 1,000), GS (1: 1,000), GFAP (1: 1,000), VEGF-A (1: 1,000), Na⁺-K⁺-ATPase (1: 10,000) or β-actin (1: 2,000), overnight at 4°C. After being washed three times with TBST, the membranes were
incubated with the corresponding secondary antibodies (1: 10,000) at room temperature for 1 hours. Followed by 3 more washes, the membranes were visualized by chemiluminescence or Odyssey infrared imaging system (LICOR Biosciences, Lincoln, NE, USA). The optical density of each band was determined by using Quantity One software (Bio-Rad), and the densitometric values for the proteins were normalized by β-actin.

**Morphology**

Intracellular edema of retinal Müller cell *in vivo* was evaluated according to the published method[19]. Briefly, the rats were killed, the eyes were enucleated and fixed in 2.5% glutaraldehyde for 30 minutes. The eyes were dissected under dissecting microscope and the anterior parts of the eye, including cornea, lens, and iris, etc, were removed. The posterior part was fixed in 2.5% glutaraldehyde for 5 more hours, which were then dehydrated in a graded alcohol series (50, 70, 95, and 100%) and embedded in epoxy resin for sectioning. Semithin sections (60 nm) were cut using an ultramicrotome (EM UC7, Leica, Germany) and stained with toluidine blue. The morphology was examined under a light microscope (Tecnai G2 20 TWIN, FEI, USA).

**Immunofluorescence**

The rat eyes were fixed at 4°C overnight in 4% PBS-buffered paraformaldehyde. The anterior segments of the eyeball were removed under the dissecting microscope. The remaining eye cups were dehydrated in 30% sucrose solution for 2 days, and then embedded in optimal cutting temperature compound (OCT; Sakura Finetek Japan Co., Ltd., Tokyo, Japan) for section. The sections (15 μm thick) were washed with PBS for five times, permeabilized and blocked in PBS buffered 1% BSA and 0.05% Triton X-100 for 1 hour. Then the sections were incubated with primary antibodies at 4°C overnight. After washed with PBS for five times, the sections were incubated with their appropriate secondary antibodies for 1 hour at room temperature in the dark. After incubation with 4’,6-
diamidino-2-phenylindole (DAPI) for 2 minutes, the slides were extensively washed five times with PBS and then mounted with coverslips. Slides were visualized with Leica microscope (DMI3000, Germany). Exposure conditions in the same channel for different groups in each experiment were consistent.

**ELISA**

The supernatants of rMC-1 cells were collected and stored at -80°C until assay. The total protein concentration in the supernatants was measured with BCA Protein Assay Kit. The concentration of VEGF-A in the supernatant was measured with ELISA kits according to the instruction of the manufacture. The VEGF-A concentration, calculated from the standard curve and normalized by the total protein concentration, was expressed as nanogram per microgram of total protein (ng/mg of total protein).

**Measurement of intracellular sodium and potassium levels**

Intracellular sodium and potassium concentration were detected with the specific indicator SBFI AM (sodium) and PBFI AM (potassium). The stock solution (1 mM) was reconstituted in DMSO, stored in dark at -20°C. The rMC-1 cells were first planted on 96-well plates and treated with glyoxal (1 mM) with or without ranibizumab (0.125 mg/mL) for 24 hours. After washed with PBS, the cells were incubated with SBFI AM or PBFI AM (diluted to the final concentration 10 μM) at 37°C for 3 hours, followed by a brief wash with PBS. Fluorescence was measured using a plate-reader (excitation = 340 nm, emission = 500 nm). The intensity of fluorescence was normalized by the cell number.

**Statistical analysis**

The results were expressed as mean ± SE. Statistical analysis was carried out with the SPSS software, version 22.0 (IBM Company, Armonk, NY, USA), and one-way ANOVA with Dunnett’s test was used. The p value of 0.05 or less was considered statistically significant.
Result

Müller cell intracellular edema was detected in diabetic rat retina, which was alleviated by ranibizumab

In order to evaluated Müller cell intracellular edema in vivo, we adopted the published method by using semithin sections of the retina[19]. As shown in Fig. 1, Compared to normal control group, the fluid accumulation was detected between nuclei of the outer nuclear layer as strip-like morphology, indicating Müller cell apical processes swollen or dilated. The edema of Müller cells was alleviated after ranibizumab treatment (Fig. 1C).

The expression of Kir4.1 was down-regulated in rat retina with diabetes progression

The examination of protein expression of Kir4.1 in diabetic rat retinas showed that, compared with the control, the Kir4.1 level in diabetic rat retinas was decreased by 21.0% in 6-week (n = 6, p > 0.05, Fig. 2A) and 46.7% in 12-week (n = 4, p < 0.05, Fig. 2B), respectively.

The decreased expression of Kir4.1 in 12-week diabetic rat retina was also confirmed with immunofluorescence. As shown in Fig. 2C, in normal control, Kir4.1 is mainly expressed in the inner limiting membrane (ILM) and co-localized with GS, a specific marker for Müller cells. However, in diabetic retinas, the distribution of Kir4.1 was largely disrupted, extending from ILM to the outer limiting membrane (OLM), with weak immunostaining especially in ILM and around retinal blood vessels.

Ranibizumab increased the expressions of Kir4.1 and AQP4 in diabetic rat retina

To test the effect of ranibizumab on the expressions of Kir4.1 and AQP4, western blot was performed in 12-week diabetic rat retinas treated with or without ranibizumab. As showed in Fig. 3A, after ranibizumab treatment, the protein level of Kir4.1 was up-regulated by 47.5% (n = 7, p < 0.05) compared with that in diabetic rat. Similarly, the protein level of
AQP4 in diabetic group was decreased significantly by 43.3% ($n = 7$, $p < 0.05$) compared to that in normal control group, which was up-regulated by 30.9% ($n = 7$, $p < 0.05$) after ranibizumab treatment (Fig. 3B). The protein expressions of GS and GFAP in the Müller cells in diabetic retinas were also evaluated and the data showed that the GS expression in diabetic retinas was decreased by 23.7% ($n = 7$, $p < 0.05$, Fig. 3C), while GFAP was increased by 222.7% ($n = 7$, $p < 0.05$, Fig. 3D), as compared with the control, indicating the activation of Müller cells with decreased function in metabolizing glutamate. However, ranibizumab has no effect on the expressions of GS and GFAP.

To further confirm the effect of ranibizumab on Kir4.1 and GFAP, we performed double immunostaining of both proteins in diabetic rat retinas treated with or without ranibizumab. As shown in Fig. 3E, in normal control, Kir4.1 was mainly expressed in the ILM and around the vessels, which co-localized with GFAP, another marker of Müller cells. However, in 12-week diabetic rat retinas, the decreased expression of Kir4.1 with its altered distribution was detected, attenuated staining pattern especially in ILM and around vessels. While GFAP immunostaining in Müller cells was increased in 12-week diabetic rat retinas with its characteristic radial immunostaining pattern. Ranibizumab treatment increased the expression of Kir4.1 as well as maintained its distribution to nearly normal level, but showed no effect on GFAP (Fig. 3E).

**Ranibizumab decreased VEGF-A and increased the protein expressions of Kir4.1, AQP4 and Dp71 in glyoxal-treated rMC-1 cells**

To further confirm above observation, we adopted glyoxal-treated rMC-1 cells to mimic diabetic condition. As shown in Fig. 4, the rMC-1 cells were treated with different doses of glyoxal (Fig. 4A) for different time points (Fig. 4B) to optimize the glyoxal treatment conditions. Cell viability was decreased dose-dependently by 0.3% (0.1 mM, $p > 0.05$), 5.2% (0.25 mM, $p > 0.05$), 15.3% (0.5 mM, $p < 0.05$), 23.4% (1 mM, $p < 0.05$) and 59.2%
(2 mM, \( p < 0.05 \)) when treated with different doses of glyoxal for 24 hours (\( n = 12 \)). When the cells were treated with glyoxal (1 mM), the cell viability was slightly increased by 2% (1 hour, \( p > 0.05 \)), then decreased by 3.1% (3 hours, \( p > 0.05 \)), 6.4% (6 hours, \( p > 0.05 \)), 16.1% (12 hours, \( p < 0.05 \)), 23.4% (24 hours, \( p < 0.05 \)) and 46.0% (36 hours, \( p < 0.05 \)) at different time points (\( n = 12 \)). Based on the result of cell viability, we chose 1 mM of glyoxal and 24 hours’ treatment for the following study.

When rMC-1 cells treated with glyoxal (1 mM) for 24 hours, the mRNA expression level of Kir4.1 was about 118.1% (\( p > 0.05 \), at 1 hour), 93% (\( p > 0.05 \), at 3 hour), 25.6% (\( p < 0.05 \), at 6 hour), 11.5% (\( p < 0.05 \), at 12 hour), 17.7% (\( p < 0.05 \), at 24 hour) of that in normal control (\( n = 6 \), Fig. 4C). The changes of Kir4.1 was also confirmed with WB, which showed that the protein level was decreased by 27.2% and 51.0%, separately, at 12 and 24 hours after glyoxal treatment (Fig. 4D).

To study the effect of ranibizumab on rMC-1 cells, glyoxal-treated rMC-1 cells were treated with or without ranibizumab and the changes of VEGF-A, Kir4.1, AQP4, Dp71 and GS were examined at both mRNA and protein levels. Although the cell viability was decreased in a time-dependent manner with glyoxal treatment (Fig. 4B), VEGF expression was increased at both 12 and 24 hours (Fig. 5A and B). The mRNA level of VEGF-A was increased by 54.4% (\( n = 6 \), \( p < 0.05 \)) and 26.4% (\( n = 6 \), \( p < 0.05 \)) at 12 and 24 hours in glyoxal-treated group (Fig. 5A). VEGF-A protein level was increased by 44.4% (\( n = 3 \), \( p < 0.05 \)) and 78.9% (\( n = 3 \), \( p < 0.05 \)), separately, at the same time points (Fig. 5B). VEGF-A level in the supernatant of cell culture was decreased significantly after ranibizumab treatment (\( n = 4 \), \( p < 0.05 \), Fig. 5C).

The mRNA levels of Kir4.1, AQP4, Dp71 and GS were also decreased significantly in glyoxal-treated group, i.e., decreased by 82.2% (Kir4.1, \( n = 8 \), \( p < 0.05 \), Fig. 6A), 71.1% (AQP4, \( n = 8 \), \( p < 0.05 \), Fig. 6D), 52.6% (Dp71, \( n = 8 \), \( p < 0.05 \), Fig. 7A) and 53.6% (GS, n
= 8, \( p < 0.05 \), Fig. 7C), respectively; which were increased by 210.4\% (Kir4.1, \( n = 8, p < 0.05 \), Fig. 6A), 65.0\% (AQP4, \( n = 8, p < 0.05 \), Fig. 6D), 36.9\% (Dp71, \( n = 8, p < 0.05 \), Fig. 7A) and decreased by 5.7\% (GS, \( n = 8, p > 0.05 \), Fig. 7C), respectively, by ranibizumab. The changes of protein expression followed a similar pattern. The protein levels of Kir4.1, AQP4, Dp71, and GS were decreased by 36.0\% (Kir4.1, \( n = 4, p < 0.05 \), Fig. 6B), 42.2\% (AQP4, \( n = 4, p < 0.05 \), Fig. 6E), 41.4\% (Dp71, \( n = 4, p < 0.05 \), Fig. 7B) and 26.9\% (GS, \( n = 4, p < 0.05 \), Fig. 7D), respectively, in glyoxal-treated group, which were increased by 39.5\% (Kir4.1, \( n = 4, p < 0.05 \), Fig. 6B), 70.5\% (AQP4, \( n = 4, p < 0.05 \), Fig. 6E), 34.9\% (Dp71, \( n = 4, p < 0.05 \), Fig. 7B) and 2.4\% (GS, \( n = 4, p > 0.05 \), Fig. 7D), respectively, after treatment of ranibizumab. The changes of Kir4.1 (Fig. 6C) and AQP4 (Fig. 6F) were also confirmed with immunofluorescence.

**Exogenous VEGF-A decreased the expression of Kir4.1 in rMC-1 cells**

To study whether the increased VEGF-A in glyoxal-treated rMC-1 cells could decrease Kir4.1 expression, we treated rMC-1 cells with recombinant human VEGF-A (rh-VEGF-A). In Fig. 8A, cell viability was increased significantly with different doses of rh-VEGF-A treatment, e.g., the cell viability increased by 29.8\% (\( n = 12, p < 0.05 \), 1 ng/mL), 32.9\% (\( n = 12, p < 0.05 \), 10 ng/mL) and 32.4\% (\( n = 12, p < 0.05 \), 100 ng/mL). The protein expressions of Kir4.1 was decreased dose-dependently by rh-VEGF-A, i.e., decreased by 23.1\% (\( n = 4, p < 0.05 \), 50 ng/mL) and 38.6\% (\( n = 4, p < 0.05 \), 100 ng/mL), indicating the down-regulation of Kir4.1 might be partially caused by increased VEGF-A in glyoxal-treated rMC-1 cells (Fig. 8B). Since ranibizumab has no effect on cell viability (Fig. 8C), the increased Kir4.1 by ranibizumab further confirmed the causal effect of VEGF-A on Kir4.1. We also detected the changes of AQP4 and Dp71 under the treatment of rh-VEGF-A and found no significant change for these 2 proteins (Data not shown).

**Ranibizumab decreased intracellular osmotic pressure by sodium efflux**
To test whether ranibizumab could prevent Müller cell from intracellular edema through decreasing the osmotic pressure, we detected the intracellular potassium and sodium level with their corresponding indicators (PBFI and SBFI). After treatment with glyoxal (1mM) for 24 hours, the intracellular potassium level is increased significantly (n=10, $p < 0.05$), while the intracellular sodium level remained relatively unchanged (n=10, $p > 0.05$) compared with that in normal control group (Fig. 9). However, when treated with ranibizumab, intracellular sodium level, but not potassium, was decreased significantly (n=10, $p < 0.05$). This result indicated that, besides up-regulation of Kir4.1, decreasing intracellular osmotic pressure might be another mechanism for ranibizumab to prevent the cellular edema of Müller cells in DR. To further explore the possible reasons, we performed the western blot to detect the protein expression of Na$^+$-K$^+$-ATPase in glyoxal-treated rMC-1 cells with or without ranibizumab treatment. The data in Fig. 9C showed that, compared with that in normal control, the expression of Na$^+$-K$^+$-ATPase in glyoxal-treated group remained unchanged, while ranibizumab treatment increased the expression of Na$^+$-K$^+$-ATPase by 20.6% (n=4, $p < 0.05$). The detailed mechanisms need further exploration.

Discussion

Diabetic macular edema (DME) is the main cause of blindness in patients with DR[20]. Anti-VEGF therapy has been an effective treatment improving both microstructure and functions of retina in DME patients. Further studying the underlying mechanisms of anti-VEGF therapy on DME and exploring other effective treatments are of great importance. In this study, we found that increased VEGF-A, and decreased Kir4.1, AQP4 and Dp71 in diabetic retinas contributed to the intracellular edema of Müller cells. Increased VEGF-A may be the initiator or causal factor for the down-regulation of Kir4.1, AQP4 and Dp71 because their altered expressions could be reversed when binding VEGF-A by ranibizumab.
The ranibizumab effect is independent of the gliotic state of Müller cells since ranibizumab showed no effect on both GS and GFAP expressions in Müller cells. Besides directly binding VEGF-A, ranibizumab could also decrease the intracellular sodium level to reduce the osmotic pressure, consequently preventing the cellular edema.

The pathogenesis of DME is complex. Breakdown of inner BRB, and dysfunction of Müller cells and RPE were all involved in the pathogenesis of DME. Müller cell, like a pump, drains the ion and water into vitreous body and retinal blood vessels with normal distribution and function of Kir4.1 and AQP4. It is reported that the distribution of Kir4.1 is altered 6-month diabetic rat retina, which is globally decreased especially in the OLM and around blood vessels[21]. Other study found Kir4.1 is absent in the perivascular areas and in ILM in 3-month diabetic rats[22]. However, most studies focused on the distributions of these channels with immunofluorescence, the protein expression levels were rarely reported in DR. In this study, we found the protein levels of Kir4.1 and AQP4 were decreased significantly in 12-week diabetic rat retina, and the immunofluorescence of Kir4.1 was greatly decreased, especially at the endfeet of Müller cells, which is consistent with the previous studies. These results indicate that the decreased expressions as well as the redistribution of Kir4.1 and AQP4 might cause the dysfunction of Müller cells, which impaired the water and ion transport in the retina, and thus caused intracellular edema of Müller cells in DR. Up-regulation of Kir4.1 and AQP4 by dexamethasone in Müller cells protected the retina from edema in a surgically induced BRB breakdown model. These data further support the pathogenesis mechanism of Kir4.1 and AQP4 in the formation of intracellular edema of Müller cell. Other studies also reported that anti-VEGF reagents could up-regulate the expression of Kir4.1 and AQP4 on primary rat Müller cells[23]. These results laid down a foundation for the treatment of DME via the up-regulation of Kir4.1 and AQP4 in Müller cells.
Ranibizumab is a recombinant, humanized neutralizing antibody fragment, directly binding all isoforms of VEGF-A. It was verified as a powerful treatment to decrease macular edema in many clinical trials[24]. Besides its binding VEGF-A, the detailed mechanisms clearing the accumulated fluid were rarely reported. We hypothesized that, except for its effect on BRB, anti-VEGF reagents might enhance the “pumping” ability of Müller cells to transport water and ion out of retina via retinal vasculature, maintaining the homeostasis of the retina. In this study, we found ranibizumab, through binding VEGF-A, protected Müller cells from edema by up-regulating Kir4.1, AQP4 and Dp71.

Ion accumulation is considered as the initial step for intracellular edema. The down-regulation of Kir4.1 could weaken efflux of potassium, causing potassium accumulation and increasing intracellular osmotic pressure. Water driven by osmotic pressure entered Müller cells through AQP4, leading to cell swelling. We found that the expression of Kir4.1, not AQP4 or Dp71, was decreased by rh-VEGF (Data not shown), indicating that the down-regulation of AQP4 and Dp71 in diabetic retinopathy might be regulated by other factors, but not VEGF.

What beyond our expectation is that the intracellular potassium level was not decreased even though Kir4.1 was up-regulated by ranibizumab. However, the intracellular sodium level was decreased significantly by ranibizumab. The possible explanation is that the activity of Na\(^+\)-K\(^+\)-ATPase was inhibited by glyoxal, which could not be reversed by ranibizumab. In this study, although we did not detect the activity of Na\(^+\)-K\(^+\)-ATPase, we did find that ranibizumab increased the protein expression of Na\(^+\)-K\(^+\)-ATPase, which might partially interpret the decrease of intracellular sodium level by ranibizumab. The detailed mechanism for ranibizumab to decrease intracellular sodium level needs further study.

Although anti-VEGF therapy is effective to treat DME, a weak correlation was reported
between gain of visual acuity and the anatomical improvement[25–28]. The loss of retinal neurons, especially cones, could result in the decreased visual acuity, which cannot be improved even after the anatomical recovery. Further the loss of retinal neurons might induce the gliotic reaction of Müller cells with overexpression of GFAP and downregulation of GS[29, 21]. In our study, cell viability and expression of GFAP and GS is not influenced by ranibizumab in vivo and in vitro, indicating that ranibizumab has no effect on the gliotic reaction. Thus, it is of importance to develop combo-therapy to treat DME, e.g., to reduce retinal edema with anti-VEGF reagents while to protected retinal neurons with neurotrophic factors or regulate the gliotic state of Müller cells.

Conclusion

Our data showed that the retinal protein expressions of Kir4.1, AQP4 and Dp71 were decreased with diabetes progression and the distributions of these proteins were also changed, causing the dysfunction of Müller cells with intracellular edema in experimental diabetic retinopathy. Ranibizumab protected Müller cells from edema via up-regulating the expressions of Kir4.1, AQP4 and Dp71 through binding VEGF-A. It could also increase the protein expression of Na\(^+\)-K\(^+\)-ATPase, thus decreasing the intracellular osmotic pressure of Müller cells. This study broadened our recognition of the mechanisms of anti-VEGF therapy for diabetic macular edema (DME) and provided clues for treating DME through targeting Kir4.1 and AQP4.

Abbreviations

AQP4
Aquaporin 4
BRB
Blood-retinal barrier
DME
Diabetic macular edema
DM
Diabetes mellitus
Dp71
Dystrophin 71
DR
Diabetic retinopathy
GFAP
Glial fibrillary acidic protein
GS
Glutamine synthetase
Kir4.1
Inwardly rectifying K+ channel 4.1
Na+-K+-ATPase
Sodium-potassium-ATPase
rMC-1
Rat Müller cell 1
RPE
Retinal pigment epithelium
VEGF: Vascular endothelial growth factor

Declarations

**Ethics approval and consent to participate**

The animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and The Guides for the Care and Use of Animals (National Research Council and Tongji University). The protocol was approved by the Committee on the Ethics of Animal Experiments of Tongji University.

**Consent for publication**

Written informed consent for publication was obtained from all participants.

**Availability of data and material**

All the data supporting our findings are provided in the manuscript.

**Competing interests**
The authors declared no conflict of interest.

**Funding**

This work was supported by grants from National Natural Science Foundation of China (81570852).

**Authors' contributions**

TQW, GTX, LL and JFZ were responsible for experimental concept and design. TQW performed most of the experiments. TQW, CYZ, HX, QXY and DDL were responsible for data acquisition and analysis. TQW drafted the manuscript, and JFZ, WYL and GTX were revised the manuscript. QP, HBT, LXL and JYX were involved in discussion. All authors reviewed and approved the final manuscript.

**References**

1. Guariguata L, Whiting DR, Hambleton I, Beagley J, Linnenkamp U, Shaw JE (2014) Global estimates of diabetes prevalence for 2013 and projections for 2035. Diabetes Res Clin Pract 103 (2):137-149. doi:10.1016/j.diabres.2013.11.002
2. Antonetti DA, Klein R, Gardner TW (2012) Diabetic retinopathy. N Engl J Med 366 (13):1227-1239. doi:10.1056/NEJMr1005073
3. Varma R, Bressler NM, Doan QV, Gleeson M, Danese M, Bower JK, Selvin E, Dolan C, Fine J, Colman S, Turpuc A (2014) Prevalence of and risk factors for diabetic macular edema in the United States. JAMA Ophthalmol 132 (11):1334-1340. doi:10.1001/jamaophthalmol.2014.2854
4. Klaassen I, Van Noorden CJ, Schlingemann RO (2013) Molecular basis of the inner blood-retinal barrier and its breakdown in diabetic macular edema and other pathological conditions. Prog Retin Eye Res 34:19-48. doi:10.1016/j.preteyeres.2013.02.001
5. Omri S, Behar-Cohen F, de Kozak Y, Sennlaub F, Verissimo LM, Jonet L, Savoldelli M,
Omri B, Crisanti P (2011) Microglia/macrophages migrate through retinal epithelium barrier by a transcellular route in diabetic retinopathy: role of PKCzeta in the Goto Kakizaki rat model. Am J Pathol 179 (2):942-953. doi:10.1016/j.ajpath.2011.04.018

6. Gardiner TA, Archer DB, Curtis TM, Stitt AW (2007) Arteriolar involvement in the microvascular lesions of diabetic retinopathy: implications for pathogenesis. Microcirculation 14 (1):25-38. doi:10.1080/10739680601072123

7. Li W, Liu X, Yanoff M, Cohen S, Ye X (1996) Cultured retinal capillary pericytes die by apoptosis after an abrupt fluctuation from high to low glucose levels: a comparative study with retinal capillary endothelial cells. Diabetologia 39 (5):537-547

8. Nguyen QD, De Falco S, Behar-Cohen F, Lam WC, Li X, Reichhart N, Ricci F, Pluim J, Li WW (2018) Placental growth factor and its potential role in diabetic retinopathy and other ocular neovascular diseases. Acta Ophthalmol 96 (1):e1-e9. doi:10.1111/aos.13325

9. Daruich A, Matet A, Dirani A, Bousquet E, Zhao M, Farman N, Jaisser F, Behar-Cohen F (2015) Central serous chorioretinopathy: Recent findings and new physiopathology hypothesis. Prog Retin Eye Res 48:82-118. doi:10.1016/j.preteyeres.2015.05.003

10. Reichenbach A, Bringmann A (2013) New functions of Muller cells. Glia 61 (5):651-678. doi:10.1002/glia.22477

11. Lange J, Hadziahmetovic M, Zhang J, Li W (2018) Region-specific ischemia, neovascularization and macular oedema in treatment-naive proliferative diabetic retinopathy. Clin Exp Ophthalmol 46 (7):757-766. doi:10.1111/ceo.13168

12. Ishii M, Horio Y, Tada Y, Hibino H, Inanobe A, Ito M, Yamada M, Gotow T, Uchiyama Y, Kurachi Y (1997) Expression and clustered distribution of an inwardly rectifying potassium channel, KAB-2/Kir4.1, on mammalian retinal Muller cell membrane: their regulation by insulin and laminin signals. J Neurosci 17 (20):7725-7735
13. Kofuji P, Biedermann B, Siddharthan V, Raap M, Iandiev I, Milenkovic I, Thomzig A, Veh RW, Bringmann A, Reichenbach A (2002) Kir potassium channel subunit expression in retinal glial cells: implications for spatial potassium buffering. Glia 39 (3):292-303. doi:10.1002/glia.10112

14. Reichenbach A, Wurm A, Pannicke T, Iandiev I, Wiedemann P, Bringmann A (2007) Muller cells as players in retinal degeneration and edema. Graefes Arch Clin Exp Ophthalmol 245 (5):627-636. doi:10.1007/s00417-006-0516-y

15. Sene A, Tadayoni R, Pannicke T, Wurm A, El Mathari B, Benard R, Roux MJ, Yaffe D, Mornet D, Reichenbach A, Sahel JA, Rendon A (2009) Functional implication of Dp71 in osmoregulation and vascular permeability of the retina. PLoS One 4 (10):e7329. doi:10.1371/journal.pone.0007329

16. Fort PE, Sene A, Pannicke T, Roux MJ, Forster V, Mornet D, Nudel U, Yaffe D, Reichenbach A, Sahel JA, Rendon A (2008) Kir4.1 and AQP4 associate with Dp71- and utrophin-DAPs complexes in specific and defined microdomains of Muller retinal glial cell membrane. Glia 56 (6):597-610. doi:10.1002/glia.20633

17. Pannicke T, Iandiev I, Uckermann O, Biedermann B, Kutzer F, Wiedemann P, Wolburg H, Reichenbach A, Bringmann A (2004) A potassium channel-linked mechanism of glial cell swelling in the postischemic retina. Mol Cell Neurosci 26 (4):493-502. doi:10.1016/j.mcn.2004.04.005

18. Rehak M, Hollborn M, Iandiev I, Pannicke T, Karl A, Wurm A, Kohen L, Reichenbach A, Wiedemann P, Bringmann A (2009) Retinal gene expression and Muller cell responses after branch retinal vein occlusion in the rat. Invest Ophthalmol Vis Sci 50 (5):2359-2367. doi:10.1167/iovs.08-2332

19. Zhao M, Valamanesh F, Celerier I, Savoldelli M, Jonet L, Jeanny JC, Jaisser F, Farman N, Behar-Cohen F (2010) The neuroretina is a novel mineralocorticoid target:
aldosterone up-regulates ion and water channels in Muller glial cells. FASEB J 24 (9):3405-3415. doi:10.1096/fj.09-154344

20. Stitt AW, Curtis TM, Chen M, Medina RJ, McKay GJ, Jenkins A, Gardiner TA, Lyons TJ, Hammes HP, Simo R, Lois N (2016) The progress in understanding and treatment of diabetic retinopathy. Prog Retin Eye Res 51:156-186. doi:10.1016/j.preteyeres.2015.08.001

21. Bringmann A, Pannicke T, Grosche J, Francke M, Wiedemann P, Skatchkov SN, Osborne NN, Reichenbach A (2006) Muller cells in the healthy and diseased retina. Prog Retin Eye Res 25 (4):397-424. doi:10.1016/j.preteyeres.2006.05.003

22. McDowell RE, Barabas P, Augustine J, Chevallier O, McCarron P, Chen M, McGeown JG, Curtis TM (2018) Muller glial dysfunction during diabetic retinopathy in rats is reduced by the acrolein-scavenging drug, 2-hydrazino-4,6-dimethylpyrimidine. Diabetologia 61 (12):2654-2667. doi:10.1007/s00125-018-4707-y

23. Gaddini L, Varano M, Matteucci A, Mallozzi C, Villa M, Pricci F, Malchiodi-Albedi F (2016) Muller glia activation by VEGF-antagonizing drugs: An in vitro study on rat primary retinal cultures. Exp Eye Res 145:158-163. doi:10.1016/j.exer.2015.11.010

24. Chen Y, Wiesmann C, Fuh G, Li B, Christinger HW, McKay P, de Vos AM, Lowman HB (1999) Selection and analysis of an optimized anti-VEGF antibody: crystal structure of an affinity-matured Fab in complex with antigen. J Mol Biol 293 (4):865-881. doi:10.1006/jmbi.1999.3192

25. Diabetic Retinopathy Clinical Research N, Wells JA, Glassman AR, Ayala AR, Jampol LM, Aiello LP, Antoszyk AN, Arnold-Bush B, Baker CW, Bressler NM, Browning DJ, Elman MJ, Ferris FL, Friedman SM, Melia M, Pieramici DJ, Sun JK, Beck RW (2015) Aflibercept, bevacizumab, or ranibizumab for diabetic macular edema. N Engl J Med 372 (13):1193-1203. doi:10.1056/NEJMoa1414264
26. Wells JA, Glassman AR, Ayala AR, Jampol LM, Bressler NM, Bressler SB, Brucker AJ, Ferris FL, Hampton GR, Jhaveri C, Melia M, Beck RW, Diabetic Retinopathy Clinical Research N (2016) Aflibercept, Bevacizumab, or Ranibizumab for Diabetic Macular Edema: Two-Year Results from a Comparative Effectiveness Randomized Clinical Trial. Ophthalmology 123 (6):1351-1359. doi:10.1016/j.ophtha.2016.02.022

27. Writing Committee for the Diabetic Retinopathy Clinical Research N, Gross JG, Glassman AR, Jampol LM, Inusah S, Aiello LP, Antoszyk AN, Baker CW, Berger BB, Bressler NM, Browning D, Elman MJ, Ferris FL, 3rd, Friedman SM, Marcus DM, Melia M, Stockdale CR, Sun JK, Beck RW (2015) Panretinal Photocoagulation vs Intravitreous Ranibizumab for Proliferative Diabetic Retinopathy: A Randomized Clinical Trial. JAMA 314 (20):2137-2146. doi:10.1001/jama.2015.15217

28. Deak GG, Schmidt-Erfurth UM, Jampol LM (2018) Correlation of Central Retinal Thickness and Visual Acuity in Diabetic Macular Edema. JAMA Ophthalmol 136 (11):1215-1216. doi:10.1001/jamaophthalmol.2018.3848

29. Izumi Y, Shimamoto K, Benz AM, Hammerman SB, Olney JW, Zorumski CF (2002) Glutamate transporters and retinal excitotoxicity. Glia 39 (1):58-68. doi:10.1002/glia.10082

Figures
Fluid accumulation was remitted by ranibizumab in diabetic rat retina. The morphology of normal control (A), diabetic (B) and ranibizumab treated (C) rat retina were shown. Arrow heads indicate the swollen apical processes. N: normal control; D12w: 12-week diabetic retina; D+R: diabetic rat treated with ranibizumab.
Expression of Kir4.1 in retina was downregulated with diabetes progression. The protein expression of Kir4.1 in 6-week (A) and 12-week (B) diabetic rat retinas.

(C) Immunostaining of Kir4.1 and GS in 12-week diabetic rat retinas (Kir4.1, green; GS, red; DAPI, blue). Data are expressed as mean ± SE (n = 6 in [A], n = 4 in [B], * p < 0.05). N: normal control; D6w: 6-week diabetic retina; D12w: 12-week diabetic retina; NC, negative control.
Figure 3

The protein changes of Kir4.1 (A), AQP4 (B), GS (C) and GFAP (D) in diabetic rat retinas treated with or without ranibizumab. (E) Co-immunostaining of Kir4.1 and GFAP in 12-week diabetic rat retinas (Kir4.1, green; GFAP, red; DAPI, blue), bar: 20 μm. Data were expressed as mean ± SE (n = 7, *p < 0.05). N: normal control; D: 12-week diabetic retina; D+R: diabetic rat treated with ranibizumab.
Figure 4

Downregulation of Kir4.1 was induced by glyoxal on rMC-1 cells. (A) Cell viability of rMC-1 cells treated with different doses of glyoxal. (B) Cell viability of 1 mM glyoxal treated-rMC-1 cells for different time points. The mRNA (C) and protein (D) expressions of Kir4.1 in rMC-1 cells treated with 1 mM glyoxal. Data are expressed as mean ± SE. (n = 12 in [A, B], n = 6 in [C], n = 1 in [D], *p < 0.05).
The expression of VEGF-A was increased in rMC-1 cells when treated with glyoxal. The mRNA (A) and protein (B) level of VEGF-A in glyoxal treated rMC-1 cells for 12 and 24 hours. (C) The concentration of VEGF-A in supernatant of glyoxal-treated rMC-1 cells with or without ranibizumab. Data are expressed as mean ± SE (n = 6 in [A], n = 3 in [B], n = 4 in [C], *p < 0.05). N: normal control; G: rMC-1 cells treated with 1 mM glyoxal; G+R: rMC-1 cells treated with glyoxal and ranibizumab.
Figure 6

The mRNA and protein change of Kir4.1 and AQP4 in glyoxal-treated rMC-1 cells with or without ranibizumab. The mRNA (A) and protein (B) expressions of Kir4.1 in glyoxal-treated rMC-1 cells with or without ranibizumab. (C) The
immunofluorescence of Kir4.1 in rMC-1 cells in 3 groups. The mRNA (D) and protein (E) expressions of AQP4 in glyoxal-treated rMC-1 cells with or without ranibizumab. (F) The immunofluorescence of AQP4 in rMC-1 cells in 3 groups. Data are expressed as mean ± SE (n = 8 in [A, D], n = 4 in [B, E], *p < 0.05). N: normal control; G: rMC-1 cells treated with 1 mM glyoxal; G+R: rMC-1 cells treated with glyoxal and ranibizumab.
Figure 7

The mRNA and protein changes of Dp71 and GS in glyoxal-treated rMC-1 cells treated with or without ranibizumab. The mRNA (A) and protein (B) expressions of Dp71 in glyoxal-treated rMC-1 cells with or without ranibizumab. The mRNA (C) and protein (D) expressions of GS in glyoxal-treated rMC-1 cells with or without ranibizumab. Data are expressed as mean ± SE (n = 8 in [A, C], n = 4 in [B, D], *p < 0.05). N: normal control; G: rMC-1 cells treated with 1 mM glyoxal; G+R: rMC-1 cells treated with glyoxal and ranibizumab.
Exogenous VEGF-A decreased the expression of Kir4.1 in rMC-1 cells. (A) Cell viability of rMC-1 cells treated with rh-VEGF-A. (B) Protein expression of Kir4.1 in rMC-1 cells treated with rh-VEGF-A. (C) Cell viability of rMC-1 cells treated with glyoxal with or without ranibizumab. Data are expressed as mean ± SE (n = 12 in [A, B], n = 4 in [C], *p < 0.05). N: normal control; G: rMC-1 cells treated with 1 mM glyoxal; G+R: rMC-1 cells treated with glyoxal and ranibizumab.
Ranibizumab decrease intracellular osmotic pressure by sodium efflux.

Intracellular potassium (A) and sodium (B) level were detected in glyoxal-treated rMC-1 cell with or without ranibizumab. (C) The protein expression of Na+-K+-ATPase in glyoxal-treated rMC-1 cells treated with or without ranibizumab. Data are expressed as mean ± SE (n = 10 in [A, B], n = 4 in [C], *p < 0.05). N: normal control; G: rMC-1 cells treated with 1 mM glyoxal; G+R: rMC-1 cells treated with glyoxal and ranibizumab.

Supplementary Files

This is a list of supplementary files associated with the primary manuscript. Click to download.

ARRIVE Guidelines .pdf
