Influences of advanced glycosylation end products on the inner blood-retinal barrier in a co-cultural cell model in vitro

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Research article

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Abstract

Background: Advanced glycosylation end products (AGEs) are harmful factors that can damage the inner blood-retinal barrier (iBRB). However, their effects on iBRB co-culture models in vitro have not been reported. This study is to understand the interactive effects of different concentrations of AGEs at different time points on rat retinal microvascular endothelial cells (RMEC) and rat retinal Müller glial cell (RMGC) co-culture models.

Methods: RMEC of Sprague-Dawley rat was isolated and cultured, identified by anti-CD31 flow cytometry and immunocytometry with von Willebrand factor polyclonal antibody. Similarly, RMGC of Sprague-Dawley rat was identified by H&E staining, and immunohistochemical method with antibodies of Glial fibrillary acidic protein (GFAP) and glutamine synthetase (GS). The transepithelial electrical resistance (TEER) value was measured with the Millicell electrical resistance system to observe the leakage of the barrier. The Transwell cell for co-cultured RMEC with RMGC were used to construct an iBRB model and tested with the addition of AGEs at final concentrations of 50 mg/L and 100 mg/L, respectively, for 24 hours, 48 hours, and 72 hours.

Results: AGEs in vitro iBRB model constructed by RMEC and RMGC co-culture led to the imbalance of VEGF and PEDF, and the permeability of the RMEC layer increased because TEER decreased in a dose- and time-dependent manner. In the AGEs intervention the vascular endothelial growth factor (VEGF) was increased, while pigment epithelial derivative factor (PEDF) decreased, respectively, in a dose- and time-dependent manner by enzyme-linked immunosorbent assay.

Conclusions: The intervention with AGEs led to change of the RMEC layer transendothelial resistance and ratio of VEGF/PEDF. The iBRB in vitro model is a good tool to study the pathogenesis of retinal vascular diseases such as diabetic retinopathy and to evaluate the candidate drugs on the diseases.

Background

The blood-retinal barrier (BRB) is a special structure in the retina that regulates the exchange of substances inside and outside the blood vessels of the retina. Human and certain species of animals have dual blood supply systems: retinal blood vessels and choroidal blood vessels. The BRB corresponding to the two vascular systems are the inner blood-retina barrier (iBRB) and outer blood retinal barrier (oBRB). iBRB is composed of retinal capillary endothelial cells, pericytes, and tightly associated with adjacent basement membranes, where astrocytes and Müller cells surround as structural scaffolding for iBRB [1]. This barrier prevents the free diffusion between the circulating blood and the neural retina, but provides nutrition to the retina, and removes endogenous organisms and foreign objects from the retina. iBRB also plays a vital role in regulating the balance of the retinal neural microenvironment, maintaining the normal metabolism, and the structural and functional integrity for preventing retinal vascular diseases such as diabetic retinopathy (DR) [2–5]. Due to the presence of BRB, the drugs into the retina is limited, which is also one of the challenges facing the development of new drugs. Therefore,

using the iBRB model would help us to understand the drug's characteristics. In vivo experiments animals can maintain the complete structure of iBRB, and the information obtained is closer to reality, but the factors affecting the experiment are not easy to control, and the operation is tedious, time-consuming and economically expensive, which is not suitable for large-scale drug screening. In recent years, more attention has been paid to the development with convenient, fast, economical and accurate in vitro models. To accurately predict how a drug will behave in vivo through an in vitro model must have as many iBRB features as possible. With an ideal in vitro model the results or conclusions may come closer to the situation in vivo, and the predictions may have more practical significance.

Since retina is a complex structure composed of many types of cells, including retinal microvascular endothelial cells (RMEC), and retinal pigment epithelial cells, neurons, retinal Müller glial cells (RMGC), pericytes, and other types of cells, an in vitro model that simulates retinal structure and function, the co-culture of different types of retinal cells with RMEC have advantages over RMEC alone. Retinal pigment epithelium and vascular endothelial cells have been cultured to study choroidal angiogenesis [6]. The certain conditioned medium was made from the extracts of retinal pigment epithelium and Müller cell co-cultures to reduce the in vitro permeability of retinal vascular endothelial cells [7]. Thus, primary retinal endothelial cells, pericytes, and astrocytes were co-cultured to mimic the blood-retinal barrier [8]. The glial cells in the vertebrate retina made of mainly RMGC play an important role in maintaining the normal structure, metabolism, and function of the retina. These glial cells that contact the vitreous cavity through the enlarged basal and the subretinal space by the microvilliact as a skeletal in the formation of the blood-retinal barrier [9], and the secretion of a variety of cytokines to regulates the permeability of the retinal barrier [10, 11]. However, most of the in vitro studies of iBRB has been based on RMEC cultured models. RMEC is the site of the early onset of DR, the damage of RMEC can lead to the collapse of iBRB, leading to the progress of DR. Therefore, RMEC culture is often used as a model for studying human pathogenesis of DR and evaluating the effects of drugs [12–14].

Advanced glycosylation end products (AGEs) from chronic hyperglycemia are involved in the occurrence and development of chronic complications of diabetes. Once AGEs are formed, they are not easily degraded. Under the condition of hyperglycemia, AGEs induce abnormal cross-linking of extracellular matrix proteins, and interact with their receptors, triggering intracellular signaling cascades that cause endothelial dysfunction [15–22]. Hyperglycemia induced by oxidative stress, hypoxia, white blood cell arrest, vasoconstriction, inflammation and angiogenesis in the retina, leads to vascular stiffness and dysfunction, pericyte apoptosis, and intraretinal destruction [23–25]. The destruction of iBRB is the early and main pathophysiological basis of retinal vascular disease caused by AGEs. The mechanism of iBRB injury is closely related to vascular endothelial growth factor (VEGF) and pigment epithelial derivative factor (PEDF). The effects of AGEs on iBRB models of rat RMEC plus RMGC co-culture in vitro need to be investigated, which would provide a certain experimental basis in vivo research for human DR and other retinal vascular diseases.

Methods

Rat RMEC isolation, culture, and identification

A single of newborn 7 days old Sprague-Dawley (SD) rat (from Laboratory Animal Center, Third Military Medical University, Chongqing, China, a written consent to use the rat was delivered) was sacrificed by cervical dislocation. The eyeballs were subsequently removed under aseptic condition, and soaked in 75% ethanol for 15 minutes, rinsed with PBS containing penicillin/streptomycin three times, and placed in an appropriate serum-free DMEM solution containing penicillin/streptomycin (Gibco, USA), and the retinas were collected under a stereo microscope. The retinas were cut into small pieces in a petri dish, digested with 0.1% type I collagenase at 37 °C for 30 minutes, and then filtered through a 200-mesh stainless steel cell sieve. The filtrates were centrifuged at 1000 rpm for 5 minutes and resuspended in DMEM medium (Gibco, USA) containing 10% fetal bovine serum. CD31 antibody-coated immunomagnetic beads (eBioscience, USA) were added and incubated at 4 °C for 30 minutes. After centrifugation at 1000 rpm for 5 minutes, the cells were resuspended in 4 ml of DMEM medium and transferred to a test tube, then placed in a magnet for 2 minutes to collect the cells. The cells were seeded into poly-L-lysine-coated plates and cultured at 37 °C in a 5% CO_2 incubator (Sanyo, Japan) after washing the magnetic beads-bound cells 4 times with 10% FBS DMEM. The culture medium was changed every other day, and the cells were passaged with 0.25% trypsin (Gibco, USA) and 0.02% EDTA (1:1) digestion solution when fused to 70–80%. The RMEC out of rat retinal vascular endothelial cells were recovered after identification by CD31/PE antibody and mouse IgG (eBioscience, USA) flow cytometry, and by using the Von Willebrand factor antibody (dilution ratio 1:400, Abcam, USA) with HRP-labeled secondary antibody at dilution 1:50 (Beyotime, China). The immunostained cells were observed with DAB and hematoxylin staining under a microscope (Olympus, Japan). All studies and processes were performed by following the Animal [Scientific Procedures] Act, 1986, and the experimental animal protocols approved by the Ethics Committee of Chengdu University of Traditional Chinese Medicine.

Rat Rmgc Isolation, Culture, And Identification

With a similar procedure until retina was gently collected under a stereo microscope. The retina was blown repeatedly to form a chyle-like mixture, which was subsequently inoculated into gelatin-coated culture flask, and the culture medium was changed after 72 hours. After the 0.25% trypsin digestion was terminated with DMEM containing FBS, the cells were collected by centrifugation at 1000 rpm for 10 minutes,and gently pipetted to make the mixture even, a drop of the cell suspension was taken to count to adjust the amount of culture medium appropriately to the cell density to 1×10^6/ml. Cells were aliquoted and routinely cultured in a 3 ml/flask in a 5% CO_2, 37 °C incubator. The cell fusion reached 80% was fixed with 4% paraformaldehyde and H&E staining, RMGC were screened and identified by glial fibrillary acidic protein (GFAP) or glutamine synthetase antibodies (GS) (Abcam, USA) immunocytochemical staining.

Construction Of In Vitro Ibrb Model

RMEC and RMGC were used to simulate iBRB models in vitro after the characterization. The third generation of primary rat RMEC was collected at 70% fusion and cultured in serum-free medium for 24 h. The second passage of rat RMGC was collected at close to 70% fusion and cultured in normal RMEC medium (DMEM containing 20% fetal bovine serum FBS and 100 U/ml penicillin + 100 U/ml streptomycin) for 24 h. In the meantime, microporous membrane was pre-coated with 1% gelatin, and single-cell suspension of RMGC was seeded at the density of 1×10^4 /cm^2 at the bottom of the plate (lower compartment), then RMEC cells were seeded at the bottom of the Transwell chamber (Corning, USA) as upper compartment at a density of $2 \times 10^4/cm^2$ after most RMGCs were attached. The model was evaluated by recording the transepithelial electrical resistance (TEER) of the RMEC layer over 3–13 days.

Teer Measurement Of Rmec Layer In Ibrb Model

TEER was measured on the RMEC layer of iBRB cell co-culture in vitro using MilliCell® ERS-2 Voltohmmeter. The measurement of each well's resistance took place at three different points selected at random. The average value Rt was recorded and the background resistance formed in the cell-free culture pool as the blank value R0 was used to calculate the resistance value of the entire endothelial cell. Under the formula of TEER = (Rt-R0) × S, where S is the effective surface area of the film. Transwells with TEER greater than $90\Omega \cdot cm^2$ were selected for further use. The TEER value is measured every other day. Decreased value of TEER indicates the increased permeability.

Enzyme-linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA) was used to determine the levels of VEGF and PEDF at presence of a final concentration of 50 and 100 mg/L of AGEs (Bioss Co., Beijing, China) for 24 hours, 48 hours, and 72 hours, respectively. After AGEs treatment in each experimental group, the cell supernatant was collected following centrifugation of 3000 rpm for 20 minutes and used for the ELISA of VEGF and PEDF (Bio-Tek, USA) according to the manufacturer's instructions. The absorbance was measured at a wavelength of 450 nm, the concentrations of VEGF and PEDF were calculated according to the linear regression equation of the standard curve and the dilution factors.

Statistical analysis

SPSS 21.0 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. All data were expressed as mean ± standard deviation. Comparisons between groups were analyzed using one-way analysis of variance and post hoc tests. Univariate analysis of variance was used for comparison of the same index among multiple groups; LSD test was used for pairwise comparison between multiple groups. All experiments for statistics analysis were repeated for 6 times. $P < 0.05$ indicated that the difference was statistically significant.

Results

Isolation and identification of rat RMEC

RMEC was isolated and cultured from newborn SD rat. As measured by CD31 flow cytometry, the purity of RMEC reached 81% (Fig. 1A). Cells were also identified using polyclonal antibody Von Willebrand factor (VWF) immunohistochemistry (Fig. 1B). These results showed that RMEC was successfully isolated and cultured because CD31 is a unique bio marker for RMEC.

Isolation And Identification Of Rat Rmgc

RMGC was isolated and cultured from newborn SD rat. H&E staining showed that the cell shape was narrow and long, the cytoplasm was abundant, the membrane was clear, the deep red large and elliptical nucleus were located in the center of the cell as mononuclear or multinucleated (Fig. 2A). More than 85% of the cells were GFAP positively expressed, a brown-yellow silk-like structure was visible in the cytoplasm (Fig. 2B) and more than 80% of the cells were positive for GS staining, especially nuclear surrounding cytosol after 5 days of culture (Fig. 2C). The results showed that RMGC was successfully characterized and cultured because GFAP and GS are bio markers for RMGC.

Changes of TEER in iBRB model after treatment with AGEs

The co-culture model of RMEC and RMGC was showed that the TEER of the RMEC layer was stable until 13 days, indicated the model was successfully established, then treated with AGEs at final concentrations of 50 mg/L and 100 mg/L, respectively for 24 h, 48 h, and 72 h, and the TEER values of the RMEC layer were measured. compared with the normal control group, the permeability of the RMEC layer of the model was increased after treatment with AGEs. The intervention with AGEs led to a decrease of TEER cross the RMEC layer in a dose- and time-dependent (Fig. 3A). Because of RMGC induced cell migration, an cell layer in vitro iBRB model made of RMEC and RMGC co-culture was established in upper compartment(Fig. 3B).

Changes of VEGF induced by AGEs in iBRB models in vitro

VEGF was measured by ELISA after treating RMEC-RMGC cell co-culture with AGEs at final concentrations of 50 mg/L and 100 mg/L, respectively for 24 h, 48 h, and 72 h. The difference of VEGF concentrations between the different experimental groups in the same period was significance ($P < 0.05$). In the low AGEs group and the high AGEs group, the VEGF levels in different periods were compared, and $P < 0.05$. The results showed that treatment with AGEs resulted in significant rise in VEGF levels in RMEC-RMGC co-culture model in a dose- and time-dependent manner (Fig. 4A).

Changes of PEDF induced by AGEs in iBRB model in vitro

Similarly, ELISA was used to determine the level of PEDF in the RMEC-RMGC cell co-culture models treated with AGEs at final concentrations of 50 mg/L and 100 mg/L, respectively for 24 h, 48 h, and 72 h. The results showed that the difference of PEDF concentrations between control and experimental groups in the either period was statistically significant, $P < 0.05$. The results showed that compared with the normal control group, treatment with AGEs resulted in a significant reduction in PEDF levels in the co-culture model in a dose- and time-dependent manner (Fig. 4B).

Changes of the ratio of VEGF/PEDF in vitro induced by AGEs

VEGF and PEDF protein concentrations were simultaneously measured with ELISA After treating RMEC-RMGC co-cultivation models with AGEs at final concentrations of 50 mg/L and 100 mg/L, respectively for 24 h, 48 h, and 72 h. The ratios of the normal control group at 24 h, 48 h, and 72 h are almost the same, and the amounts of VEGF and PEDF were in a relatively balanced state. However, both low AGEs group and the high AGEs group were higher than those in the normal control group. The ratio of low AGEs group and high AGEs group increased at 48 h and continued to increase at 72 h. The results showed that compared with the normal control group, the treatment of AGEs caused VEGF proportionally increased in the co-culture, which means lead to the inhibition of PEDF in a time-dependent manner (Fig. 5).

Discussions

It has been reported that Müller cells induce a barrier in vascular endothelial cells [26], and Müller cells play an important role in the formation of retinal vascular barrier. Therefore, RMGC plays an active role in the induction and maintenance of RMEC transition from non-barrier cells to barrier cells and in vitro models produced by Müller cells that mediate the barrier-promoting and barrier resistance effects on RMEC [27]. Studies have shown that the most important pathological basis and morphological changes in early DR start in iBRB, and the structure of iBRB will be damaged if directly exposed to hyperglycemia [28]. A large number of studies have shown that AGEs are widely present in various eyeball tissues such as the cornea, retina, vitreous, lens, Bruch's membrane, sclera, and optic nerve, directly or indirectly leading to a series of eye diseases including retinopathy [29, 30]. AGEs not only cause platelet activation and aggregation but also stimulate prothrombin activity by increasing the expression of tissue factors, which lead to thrombosis [31]. In the meantime, AGEs shown to inhibit the production of prostacyclin and induce the production of plasminogen activator inhibitor-1 in endothelial cells through interaction with the receptor of AGE (RAGE) [32]. Therefore, AGEs may cause platelet aggregation and fibrin stabilization leading to a tendency of thrombosis, thereby promote vascular damage in diabetes. AGEs may also induce apoptosis and dysfunction of various cells in the retina and promote the process of DR [33]. In experimental DR model rats, accumulated AGEs directly affect the normal structure and function of Müller cells [34], but AGEs blockers can reduce the progression of DR [35]. Our RMEC and RMGC co-

culture model may be better than RMEC cultured alone. We demonstrated that co-cultured RMEC and RMGC were stabilized because TEER remained higher than $90\Omega \cdot cm^2$ after the 11th day, which indicated that the barrier was successfully established. With the established iBRB model, the addition of AGEs with different final concentrations led to iBRB damage, and this was indicated by the reduced TEER value.

Due to the large accumulation of AGEs, iBRB can be easily destroyed, and DR aggravates resulting in vascular edema and nerve tissue damage, eventually lead to vision loss [36]. Overexpression of VEGF under pathological conditions causes increased vascular permeability and neovascularization. At present, a large number of clinical and animal experiments have shown that the high glucose environment in blood and tissues stimulates the large-scale production of VEGF is one of the mechanisms of DR development, and high levels of VEGF cause retinal iBRB damage and subsequently retinal exudation, hemorrhage, edema, and neovascularization, and early diabetic mellitus retina. VEGF and PEDF are a group of major cytokines that are most closely related to the occurrence and development of DR. Therefore, the cytokines VEGF and PEDF were tested separately and combinedly for analyzing the balanced relationship in this experimental model. In the normal eye, Müller cells, pericytes, pigment epithelial cells, and endothelial cells of the retina secrete VEGF, but the expression levels are low. The low secretion is conducive to the maintenance of normal blood vessel function. In this experiment, the VEGF concentration between experimental groups in the same period was increased from the low AGEs group to high AGEs group at the different periods ($P < 0.05$), and our observation is consistent with previous related studies, the higher of the AGEs concentration, the higher of VEGF [37, 38].

PEDF is a polypeptide mainly from retinal pigment epithelium and retinal Müller cells in the eye. The main feature of this factor is that it inhibits vascular leakage and vascular regeneration. A large number of studies have shown that the expression of PEDF in the eyes patients with DR is significantly reduced, and this situation is more pronounced in patients with proliferative DR. Both cell culture and animal experiments have confirmed that the pericytes cultured with AGEs have significantly improved their functions after adding exogenous PEDF. However, the accumulation of AGEs in diabetic mellitus significantly inhibits the expression of PEDF mRNA, and the transcription and expression of PEDF are reduced [39]. The oxidative stress aggravates injury in a high glucose environment, promotes damage to the iBRB structure, and thereby accelerates retinal edema and the formation of new blood vessels [40]. In this experiment, the PEDF concentrations between control and experimental groups in the same period was significantly different, the difference of PEDF in low AGEs and high AGEs groups at different period was also significant. This shows that the higher AGEs concentration and the longer time lowered concentration of PEDF.

The mechanism of iBRB injury under diabetic pathological conditions is related to VEGF and PEDF. VEGF promotes early iBRB injury in DR and increase the leakage of diabetic retinopathy vessels; on the other hand, PEDF has the function of inhibiting vascular leakage of DR with a protective effect on iBRB. Under physiological conditions, there is a balanced relationship between PEDF and VEGF. PEDF inhibits the increase of VEGF's vascular permeability and angiogenic potential [41]. In this experiment, we showed that the ratio of the normal control group at different time points was almost the same, indicating that the

protein levels of VEGF and PEDF are in a relatively balanced state. But the ratio of VEGF/PEDF changed at the low AGEs group and the high AGEs group at different time points, both increased at 48 hours and continued to increase at 72 hours. This shows that the balance of VEGF and PEDF was lost after the addition of AGEs, and the expression ratio in vitro revealed a similar in vivo expression pattern.

Conclusions

This experiment successfully established an iBRB cell co-culture model in vitro with permeability changes of iBRB by indirectly measurement of TEER and treated the model with AGEs to simulate iBRB damage, and to detect changes of VEGF and PEDF. Our findings showed that VEGF levels increased in the cell co-culture model, PEDF levels decreased, both in a dose- and time-dependent manner, and the balance of VEGF and PEDF was lost at presence of AGEs; The increase of permeability is dependent on the dose and time of AGEs intervention, so the imbalance of VEGF and PEDF may be one of the important reasons for the increase in permeability of RMEC layer. Thus, in vitro iBRB model constructed by RMEC and RMGC co-culture can be usedl for studying the pathogenesis of retinal vascular diseases such as DR and for evaluating the impact of candidate drugs, even though the relevent in vivo assays remain needed.

Abbreviations

AGEs:advanced glycosylation end products; iBRB:inner blood-retinal barrier; RMEC:retinal microvascular endothelial cells; RMGC:retinal Müller glial cell; GFAP:Glial fibrillary acidic protein; TEER:transepithelial electrical resistance; VEGF:vascular endothelial growth factor; DR:diabetic retinopathy; SD:Sprague-Dawley; ELISA:Enzyme-linked immunosorbent assay.

Declarations

Ethics approval and consent to participate

All studies and processes were performed by following the Animal (Scientific Procedures) Act 1986, and the experimental animal protocols approved by the Ethics Committee of Chengdu University of Traditional Chinese Medicine, Chengdu, China. All participants involved were informed of the purpose of this study and a written consent was obtained from themselves.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article. **Competing interests**

The authors declare that they have no competing interests.

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Authors' Contribution

All authors have read and approved the manuscript. XJX, MZ, Research Design, Data Interpretation and manuscript preparation, CY, experiment and manuscript preparation, YM, JY, literature and clinic studies.

Acknowledgments

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Figures

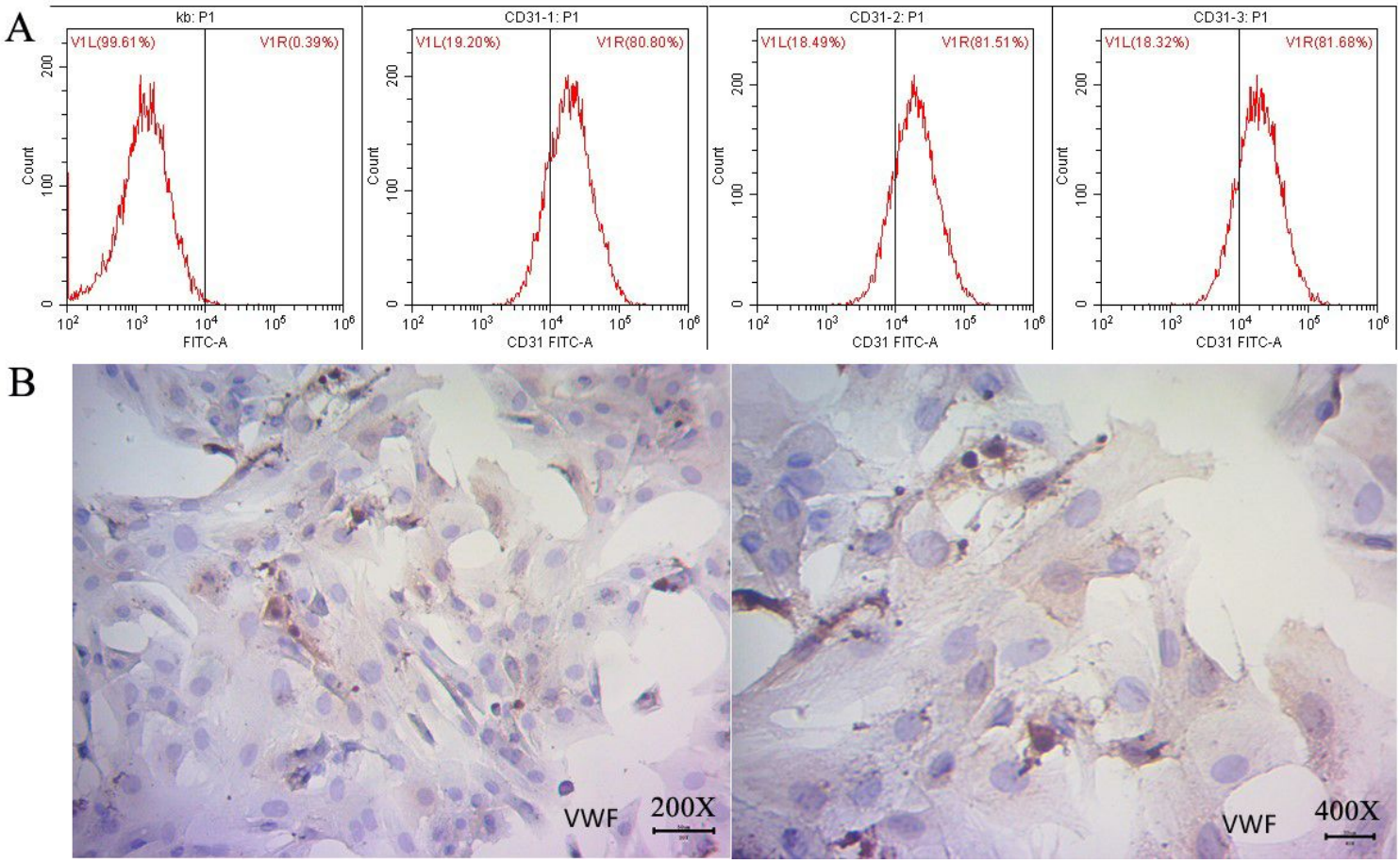

Figure 1

Isolation and identification of RMEC from rat retinal vascular endothelial cells (RRVEC). RRVEC was isolated and cultured from the retina of 7-day-old neonatal SD rat, and RMEC was verified after three passages and flow cytometry. A: Verification of RMEC using flow cytometry with CD31 antibody. Kb: the blank control without antibody. P1, the cell content after removal of cell debris. V1L, the content of the negative cells which was not labeled by CD31; V1R, the content of the positive cells which were labeled by CD31, RMEC >80% among RRVEC. B: Immunological verification of RMEC with a polyclonal antibody against the Von Willebrand factor.

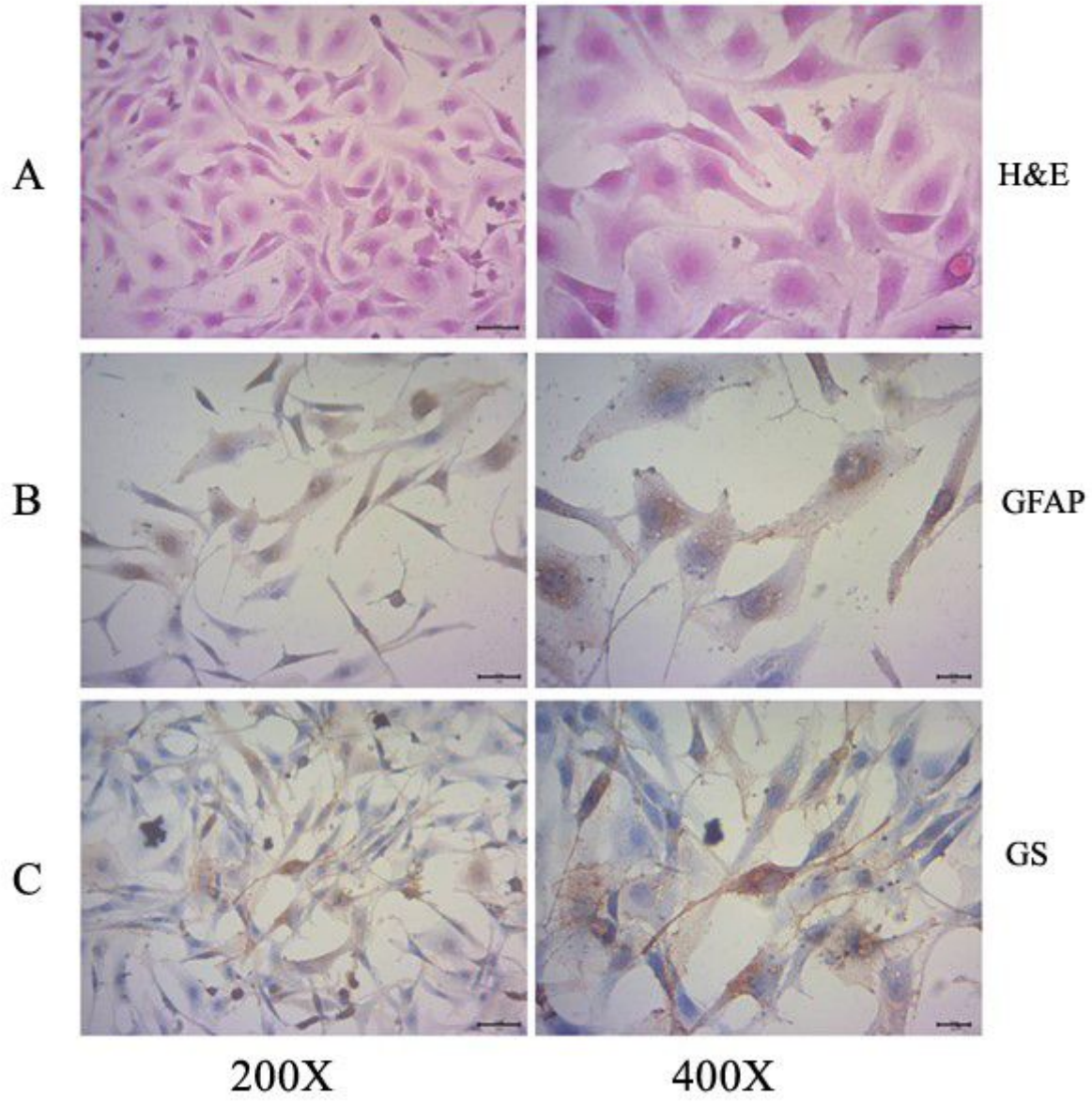

Figure 2

Isolation and identification of RGMC from rat retinal Müller cells (RRMC). RRMC was isolated and cultured from the retina of 7-day-old newborn SD rat, and RGMC was verified using A: H&E staining, B: GFAP and C: GS immunocytochemical staining after two passages. RGMC among RRMC was indicated with GFAP and GS positive.

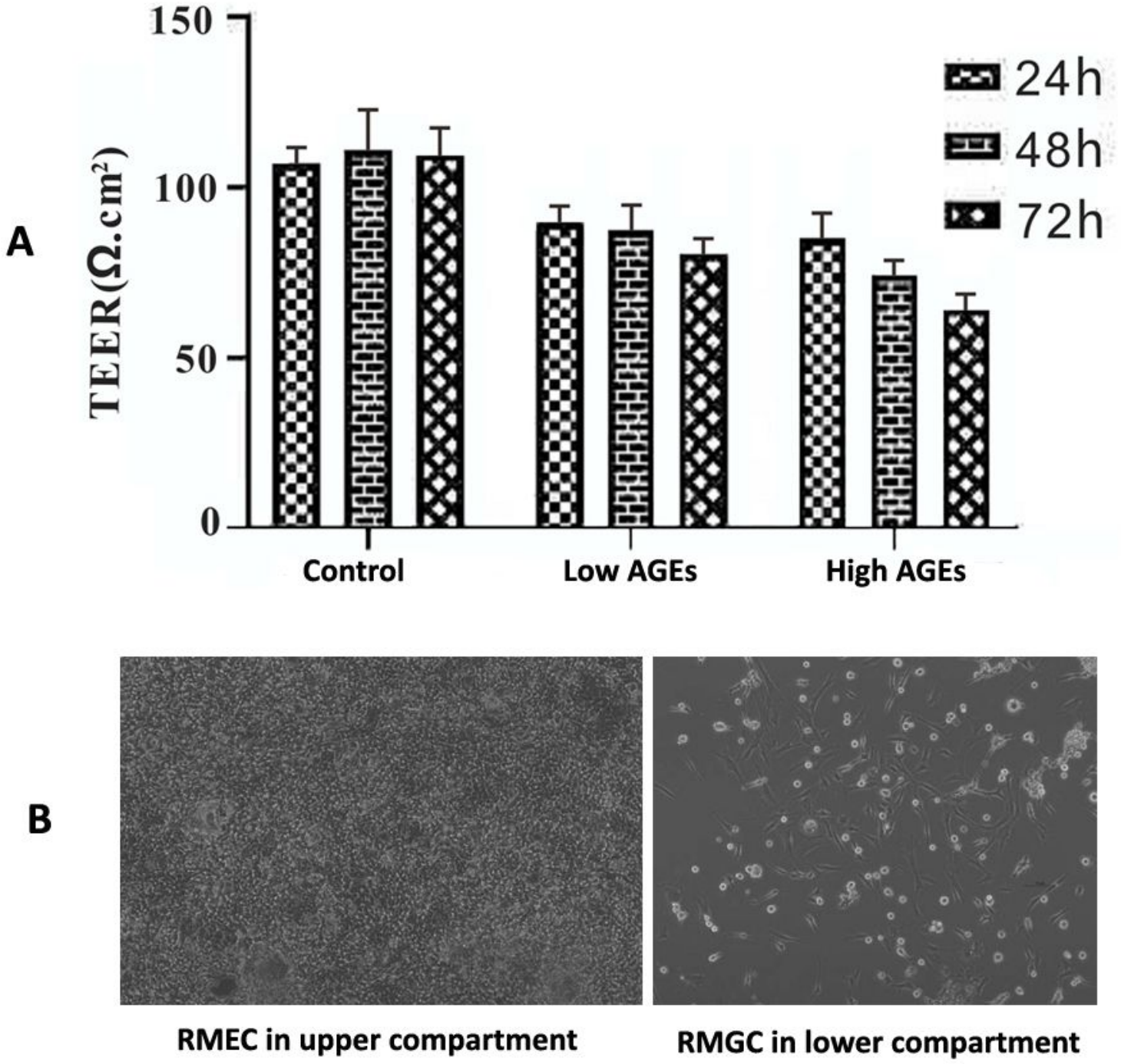

Figure 3

Change of TEER in RMEC layer of in vitro iBRB-Müller cells co-culture byAGEs. A: The TEER of RMEC layer of in vitro iBRB-Müller cells co-culture model was determined after treatment with indicated groups for 24h, 48h, and 72h. B: Transwell migration assay, RMGC induced barrier-like Co-cultured RMEC and RMGC cells (100X).

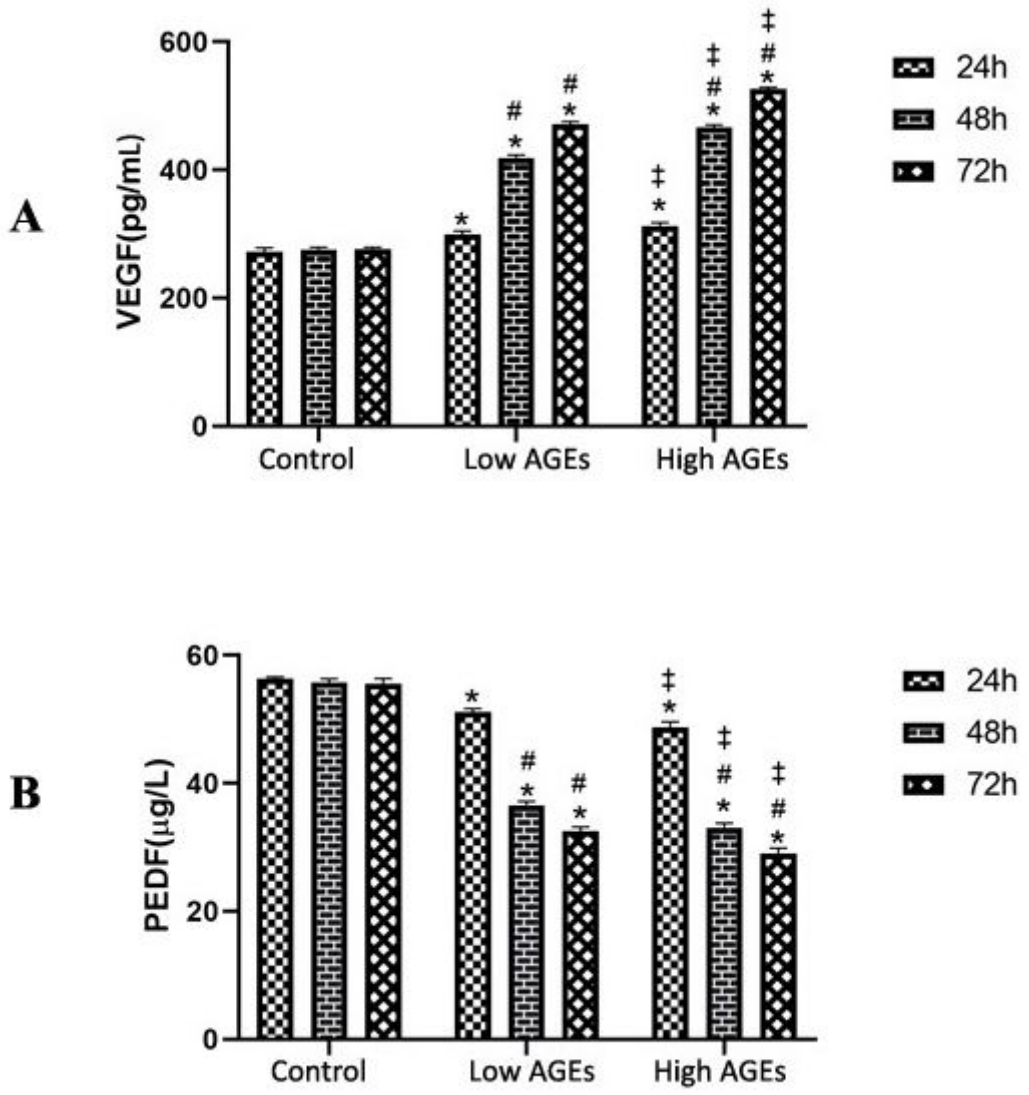

Figure 4

Changes of VEGF and PEDF in iBRB-Müller cells co-culture by AGEs. A: VEGF in the media of in vitro iBRB-Müller cells co-culture model were determined using ELISA after treatment with indicated AGEs for 24h, 48h, and 72h. *Compared with the vehicle control group, $P < 0.05$; # compared with the low dose at 24 h earlier, $P < 0.05$; ‡ compared with the high dose group, $P<0.05$, respectively, at the same time point. B: PEDF in the media of in vitro iBRB-Müller cells co-culture model were determined using ELISA after treatment with indicated AGEs for 24h, 48h, and 72h. *Compared with the vehicle control group, $P < 0.05$; #compared with the low dose at 24 h earlier, $P < 0.05$; ‡compared with the high dose group, $P < 0.05$, respectively, at the same time point.

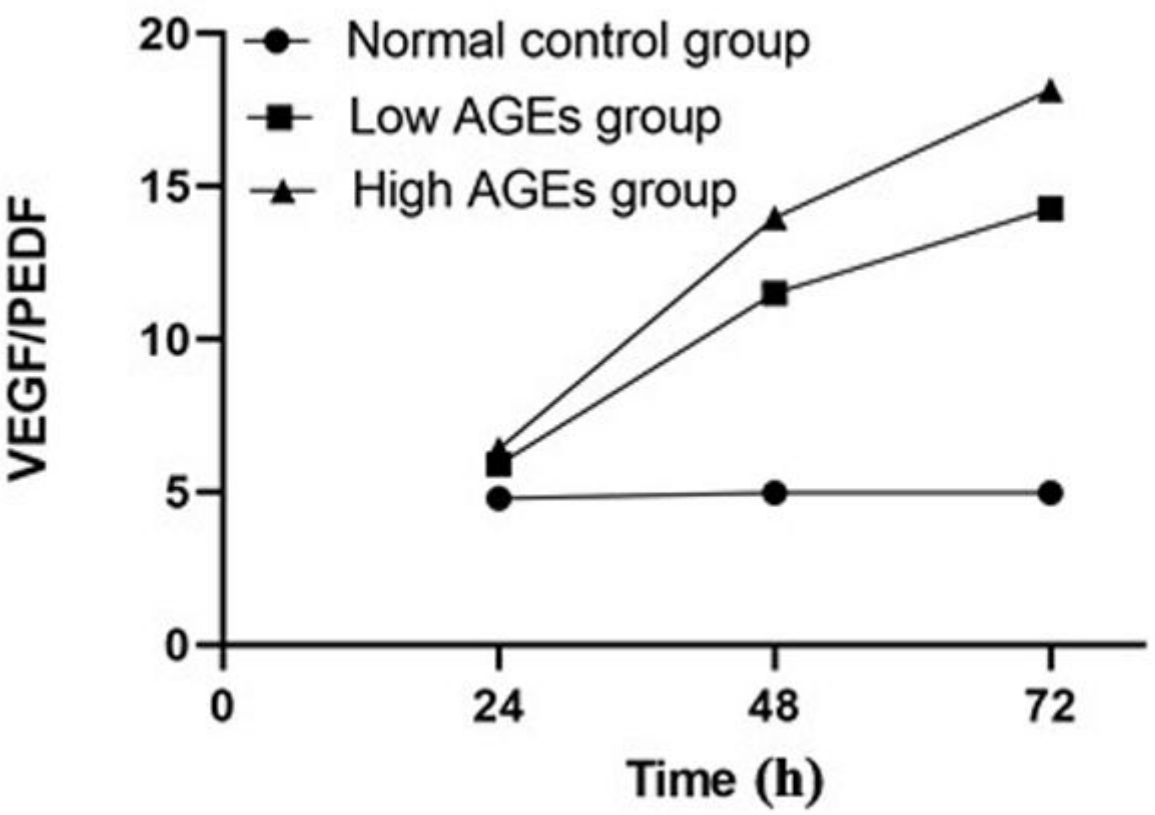

Figure 5

VEGF/PEDF in RMEC layer of in vitro iBRB-Müller cells co-culture after AGEs treatment. VEGF and PEDF in the media of in vitro iBRB-Müller cells co-culture were determined simultaneously using ELISA after treatment with indicated AGEs for 24h, 48h, and 72h, and the ratio of the average values of the two cytokines were plotted against time points.

Supplementary Files

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