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Original Article

FK506 alleviates blood-retinal barrier breakdown and reduces retinal vascular permeability in early streptozotocin-induced diabetic rat

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Abstract: Objective: Diabetic retinopathy is a complex disease that potentially involves inflammation in its pathogenesis. It has been demonstrated that FK506, one of its potent immunosuppressive agents, possess anti-inflammatory properties. Therefore, the present study aimed to explore whether FK506 could reduce vascular damage in experimental diabetic retinopathy. Methods: The streptozotocin-induced model of diabetes was established using Wistar rats. Animals received single intravitreal injections of FK506 or vehicle after seven days. The blood retinal barrier (BRB) was determined using Evans blue permeation, leukostasis was determined using Fluorescein-isothiocyanate (FITC)-coupled Concanavalin A lectin, and the expression of VEGF and ICAM-1 was detected by western blot. Results: Intravitreal delivery of FK506 protects BRB integrity against vascular leakage by inhibiting diabetic retinal leukocyte adhesion to retinal vessels and downregulating the expression of VEGF during early diabetes. Twenty-four hours after FK506 treatment, VEGF decreased by 82.17%, compared with diabetic rats without treatment ($P<0.01$). In addition, FK506 inhibited the expression of ICAM-1, a significant inflammation protein. Moreover, ICAM-1 decreased by 74.27% after treatment with 2.5 ng of FK506, when compared with diabetic animals without treatment ($P<0.01$). Conclusion: FK506 could inhibit the degree of retinal inflammation and attenuate neovascularization in early diabetic mice. The topical application of FK506 appears as a highly promising novel approach for the treatment of diabetic retinopathy.

Keywords: Diabetic retinopathy, blood retinal barrier, ICAM-1

Introduction

Diabetic retinopathy (DR), the most common microvascular complication of diabetes mellitus, is the leading cause of new blindness in the working population, worldwide [1]. A previous study has shown that the number of people with diabetes was nearly 366 million in 2011, and this may increase to 552 million by 2030 [2]. Diabetes impairs vision, and finally causes blindness as a result of long-term accumulated damage to the small blood vessels in the retina. Any clinical symptom of retinopathy would take several years before appearing in diabetic patients due to gradual but accelerating deterioration. Unfortunately, no known treatments, specifically for mild to moderate DR, have been found at present. Therefore, the implementation of treatments should immediately start before DR progresses to the irreversible stage. To date, the detailed mechanism of DR remains not fully understood. Therefore, there is a significant unmet need to understand the pathogenesis of diabetic damage, and develop novel and effective therapies to prevent diabetes-related vision loss. Increasing evidences have shown that inflammation plays a considerable role in the development of DR [3]. In addition, a large number of studies have demonstrated that the mechanism underlying increased risk of cardiovascular disease in subjects with diabetes inflammation and markers of inflammation correlates with the incidence of diabetes [4]. Animal studies have shown that if the inflammatory cascade at any of its multiple steps was inhibited, the histopathology characteristic of the early stages of DR can be inhibited [5]. In recent years, numerous clinical and

laboratory investigations have identified increased vascular permeability and leukostasis during the initial events of DR [6-8]. The upregulated expression of intercellular adhesion molecule-1 (ICAM-1) on vascular endothelial cells leads to leukocyte adhesion to the vascular endothelium and the accumulation of leukocytes within the retina [9, 10]. In addition, studies have shown that leukocytes cause capillary occlusion, endothelial cell apoptosis, and finally blood-retinal barrier breakdown (BRB) when adherent to the vascular endothelium [11]. Prior to the clinical identification of DR, these pathological changes initially remain unnoticed. Then, various clinical symptoms appear such as areas of nonperfusion, retinal hemorrhage and retinal edema; and finally, serious loss of visual acuity occurs.

Tacrolimus (FK506), a potent immunosuppressive agent, has been widely used in transplantation, and in treatments for rheumatoid arthritis and atopic dermatitis [12-16]. Furthermore, it has a definite effect on immunorelated ocular diseases. Moreover, it has antioxidant and anti-inflammatory effects on transient focal cerebral ischemia [16]. Therefore, we hypothesized that FK506 might be effective on attenuating damages occurring in the blood vessel wall of the retina in diabetes.

Materials and methods

This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Diabetes model and animal groups

Male Wistar rats, weighing 180-200 g (Animal Experimental Central, Shanxi University, China), were used in this study. Diabetes was induced with a single intraperitoneal injection of streptozotocin (60 mg/kg, Sigma) in 10 mM of citrate buffer (pH 4.5) after 24-hour fasting. Animals that served as non-diabetic controls received an equivalent amount of water alone. Streptozotocin-injected rats with blood glucose levels >250 mg/dl were deemed diabetic after 24 hours. Blood glucose levels were measured again to confirm the diabetic status just before the experiment. The animals were randomly divided into three groups: (1) non-diabetic controls (control group), (2) untreated diabetes administered with equal amounts of water (diabetic group), and (3) diabetics administered with 2.5 ng of FK506 (FK506 group). Seven days after diabetes was induced, drugs (Sigma-Aldrich, St. Louis, MO, USA) or vehicle were administered by injection in both vitreous cavities using a 30-G needle. After 24 hours, these experimental animals were sacrificed.

Transmission electron microscopy (TEM)

The samples were cut into 1 mm^3 pieces, fixed with 4% glutaraldehyde for one or two hours, and washed with phosphate-buffered saline three times for 10-15 minutes. Then, the samples were fixed again with 1% osmium tetroxide for an hour, followed by washing with phosphate-buffered saline three times for 15 minutes. Then, these samples were dehydrated using acetone (50%, 70%, 80%, 90% and 100%) three times for 10-15 minutes. Subsequently, the samples were soaked with EPON812 and acetone (1:1) for one hour, EPON812 and acetone (3:1) for three hours, and EPON812 for 12 hours. The samples were embedded and labeled before aggregation at 37°C for 12 hours, 45°C for 12 hours, and 60°C for 24 hours. After slicing, the slices were stained with lead nitrate for 10-20 minutes and uranyl acetate for 20-30 minutes before the integrity of the nerve fibers was observed under a transmission electron microscope (H-600, Hitach, Japan).

Measurement of blood-retinal barrier permeability

BRB permeability was quantified using Evans blue, which binds to the plasma albumin, using the method described by Xu *et al.* [17] with some modifications. Under anesthesia, Evans blue (45 mg/kg) was administered to rats *via* the iliac vein; and were kept on a warm pad for 120 minutes. After perfusion to remove the dye from the vessels, the eyes were enucleated and the retinas were isolated. The wet weight of each retina was measured. Evans blue was extracted by incubating each retina in 0.12 ml of formamide for 18 hours at 70°C. The extract was centrifuged at 70,000 g for 45 minutes at 4°C. The absorbance of the filtrate was measured at 620 nm and 720 nm. The dye concentration was calculated from the standard curve of Evans blue in formamide and normalized to retina weight.

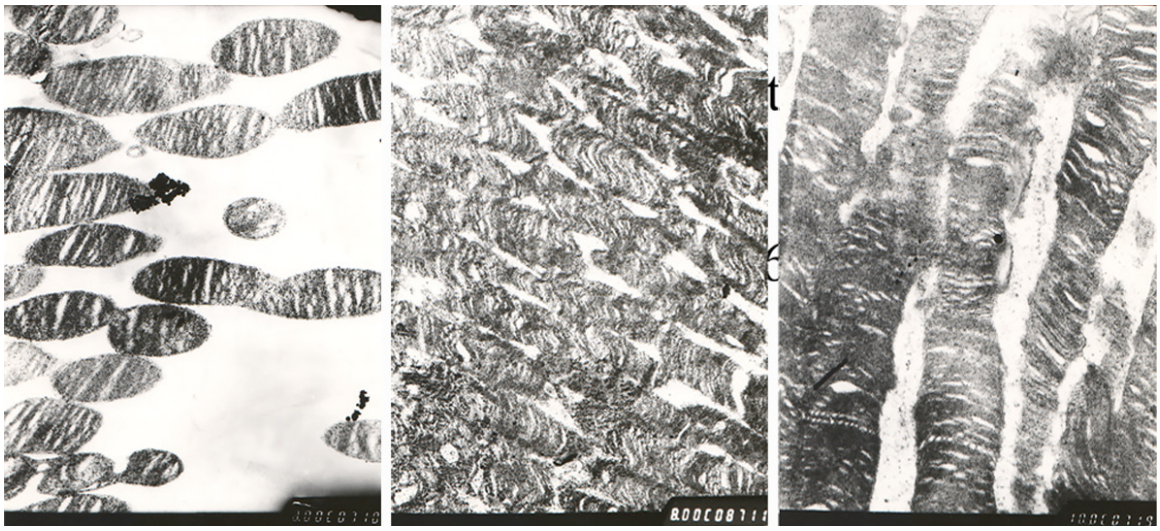

Figure 1. Treatment with FK506 reduced the damaged structures in rat retina induced by diabetes. Representative Transmission electron microscopy images from the three groups.

Visualization of retinal vessel leakage

Retinal vascular leakage was visualized using Evans blue, as previously described [17]. Under anesthesia, Evans blue (45 mg/kg) was administered in rats *via* the iliac vein, and were kept on a warm pad for 120 minutes. The retina was isolated, flat-mounted and examined under a confocal microscope (LSM 510; Carl Zeiss, Gottingen, Germany) to check for Evans blue extravasation from retinal vessels.

Evaluation of leukocyte adhesion to retinal vessels

The retinal vasculature and adherent leukocytes were labeled with Fluorescein-isothiocyanate (FITC)-coupled Concanavalin A lectin (conA) (Vector Laboratories, Burlingame, CA) [18, 19]. After 24 hours, the experimental animals were anesthetized and the chest cavity was opened carefully to introduce a perfusion needle into the left ventricle. Erythrocytes and non-adherent leukocytes were removed by PBS perfusion through the right atrium, followed by the perfusion of FITC-coupled conA (40 mg/ml in PBS; pH 7.4; 5 mg per kg body weight) to label adherent leukocytes and vascular endothelial cells. Then, PBS perfusion was performed to remove the residual unbound lectin. The retinas were carefully removed and fixed with 1% paraformaldehyde. Images of the flat mounts were obtained using fluorescence microscopy (Axiovert100; Carl Zeiss, Germany). Adherent leukocytes and obliterated retinal capillaries were counted in four quadrants of the mid-retina.

Western blot analysis

Animals were sacrificed with an overdose of anesthesia, and the retinas were immediately isolated. The retinas were homogenized in lysis buffer and centrifuged at 4°C for 10 minutes. The supernatant was collected and mixed with sample buffer. Samples (each with 100 mg of total protein) were boiled for three minutes, separated by SDS-PAGE, and electroblotted onto a polyvinylidene difluoride membrane (BioRad). Nonspecific binding was blocked with 5% normal goat serum, and the membranes were incubated at room temperature for two hours with mouse anti-VEGF (1:1,000 dilution), rabbit anti-ICAM-1, or mouse anti-β-actin; followed by incubation with horseradish peroxidase-conjugated goat antibody to rabbit immunoglobulins. The quantification of densitometry was performed using the NIH Image program (developed by Wayne Rasband; National Institutes of Health, Bethesda, MD).

Statistical analysis

Data was expressed as mean ± standard deviation (SD). Datas from diabetes increases BRB permeability: protective effect of FK506, FK-506 inhibits the expression of VEGF in diabetic rat retinas, treatment with FK506 reduced the leukocyte attachment and FK506 inhibits the

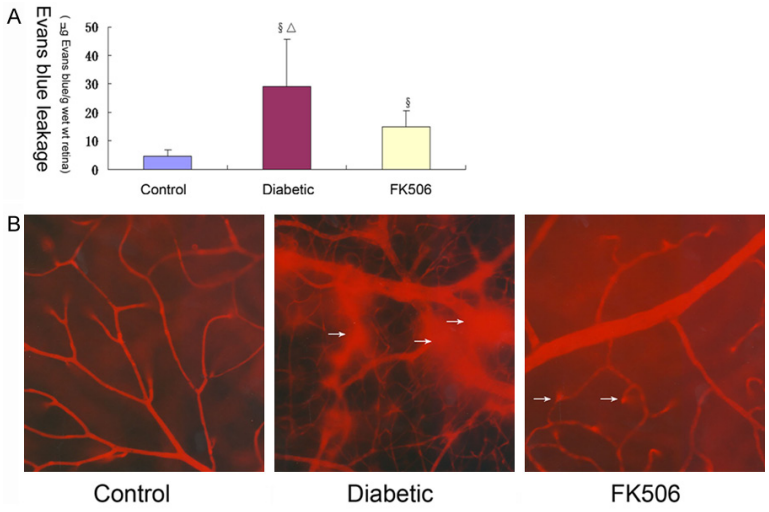

Figure 2. Diabetes increases BRB permeability: protective effect of FK506. A: Quantitative measure of BRB permeability by quantification of extravasated Evans blue. Data are presented as g of Evans blue per retina wet wt (g), and represents the mean ± SEM of 7-10 animals. $^{§}P<0.05$, *vs.* the control group; $^{Δ}P<0.05$, *vs.* the FK506 group. B: Representative images showing Evans blue fluorescence, allowing the detection of leaking sites (arrows) in retinal vessels. In the retina of control animals, Evans blue fluorescence was limited to the blood vessels, while in diabetic retinas, the dye leaks out of the vessels to the retinal tissue. FK506 treatment prevents the leakage of Evans blue.

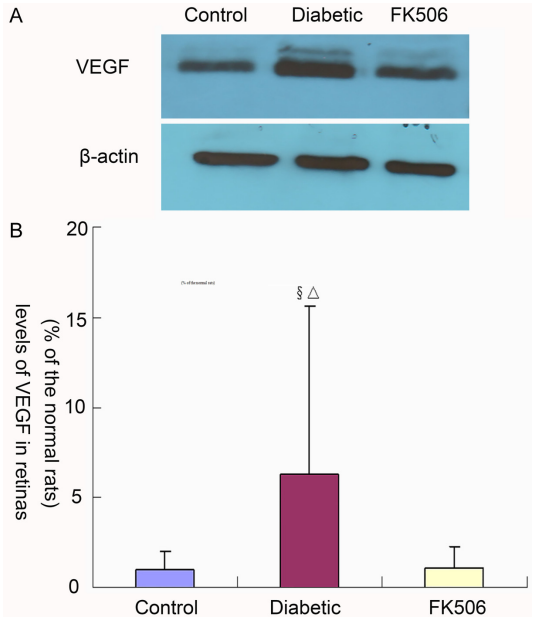

Figure 3. FK506 inhibits the expression of VEGF in diabetic rat retinas. The protein levels of VEGF were evaluated in whole rat retinal extracts by western blot. Data are presented as the percentage of controls, and represents the mean ± SEM of 6-7 animals. $^{§}P<0.05$, *vs.* the control group; $^{Δ}P<0.05$, *vs.* the FK506 group.

expression of ICAM-1 in diabetic rat retinas were analyzed with one-way analysis of variance, followed by Dunnett's test, wherever appropriate. A *P*-value<0.05 was considered statistically significant.

Results

Effects of FK506 on blood glucose in diabetes rats

Blood glucose levels (mg/dl) in diabetic animals markedly and steadily increased after the induction of diabetes (353.5 ± 15.3 mg/dl), when compared with controls) (95.3 ± 8.7 mg/dl). At the same time, blood glucose levels in diabetic rats treated with FK506 (348.7 ± 16.4 mg/dl) were found to be similar to values in diabetic animals without treatment.

Effects of FK506 on damaged structures in rat retina induced by diabetes

In order to observe the structural changes in the retina induced by diabetes, TEM was employed. TEM images revealed that the outer segment membrane was mainly disordered in the diabetic group. Furthermore, the gap widened, the outer core layer was swollen, the nuclear circumferential gap widened, the nuclear chromatin gathered, and electron density increased (**Figure 1**). Moreover, damage in the outer segment membrane was alleviated in the FK506 group compared with the diabetic group.

Effects of FK506 on diabetes-induced BRB permeability

Diabetes notably increased BRB permeability in diabetic rats when compared with control animals (29.2 ± 16.4 *vs.* 4.5 ± 2.3 μg of Evans blue/g wet wt retina, *P*<0.001). In diabetic rats treated with FK506, a significant decrease in BRB permeability (14.9 ± 5.6 μg of Evans blue/g wet wt retina) was observed when compared with diabetic animals without treatment (**Figure 2A**).

In order to obtain a morphological insight into the permeability change of the retinal vascular network induced by diabetes, Evans blue angiography was used to measure blood vessel leakage in retina flat mounts. Under the excita-

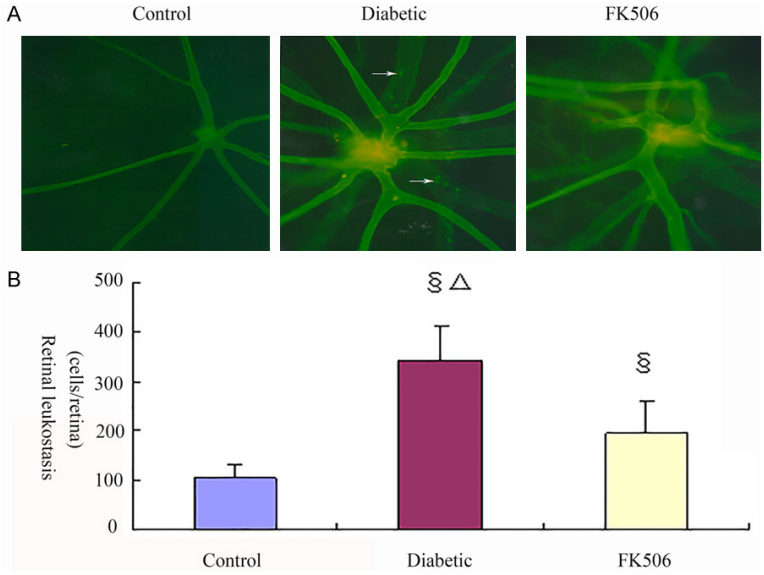

Figure 4. Treatment with FK506 reduced the leukocyte attachment. Con A perfusion retinas show attached leukocytes (arrows) within the vasculature of diabetic retinas. A. Representative retina flat-mount images from three groups. B. Quantitative analysis of leukocyte adhesion. $^{§}P<0.05$, *vs.* the control group; $^{\Delta}P<0.05$, *vs.* the FK506 group.

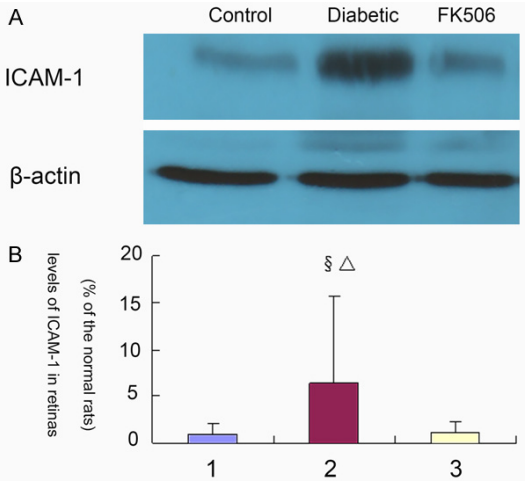

Figure 5. FK506 inhibits the expression of ICAM-1 in diabetic rat retinas. ICAM-1 protein levels were evaluated in whole rat retinal extracts by western blot. Data are presented as the percentage of controls, and represents the mean ± SEM of 6-7 animals. $^{§}P<0.05$, *vs.* the control group; $^{\Delta}P<0.05$, *vs.* the FK506 group.

tion of green light, the retinal capillarity network was clearly displayed with red fluorescence, which revealed the fluorescence leakage around the laser spots. However, Evans blue fluorescence was limited to the blood vessels in control retinas. Furthermore, focal leakage of the dye from the capillaries and larger vessels was detected in diabetic animals. However, FK506 treatment prevented this change (**Figure 2B**), which corroborated the data obtained with the quantitative Evans blue assay.

Effects of FK506 on VEGF expression in diabetic rat retinas

Retinal vascular dysfunction caused by VEGF is the major pathological change that occurs in diabetic retinopathy. In the retinas of diabetic animals, the protein content of VEGF increased to 6.13 folds higher than that in the control group. After 24 hours from FK506 treatment, an 82.17% decrease in VEGF was detected when compared with diabetic rats without treatment ($P<0.01$, **Figure 3**).

FK506 inhibits leukocyte adhesion to retinal vessels induced by diabetes

In order to examine the effects of FK506 on adherent diabetes-induced leucocytes and on the breakdown of the BRB, non-attached blood cells were removed by vascular perfusion. Then, the perfusion of Con A was performed to identify the attached leukocytes using fluorescence microscopy imaging (**Figure 4A**). An increase in the number of leukocytes that adhered to retinal vessels (38 ± 4 leukocytes/animal) was found in diabetic animals when compared with that in the control group (16 ± 2 leukocytes per animal). After 24 hours from FK506 treatment, a 31.58% decrease in the number of adhering leukocytes was detected when compared with diabetic animals without treatment ($P<0.01$, **Figure 4B**).

FK506 prevents the upregulation of ICAM-1 levels in diabetic rat retinas

The adhesion of leukocytes to retinal vessels is mainly mediated by the interaction with ICAM-1, which is expressed in retinal endothelial cells. The protein content of ICAM-1 in the retinas of diabetic animals increased to 136.4 ± 12.5% of that in controls. However, FK506 treatment inhibited the diabetes-induced upregulation of ICAM-1 (**Figure 5**). Furthermore, a 74.27% decrease in ICAM-1 was detected after

treatment with 2.5 ng of FK506, when compared with diabetic animals without treatment ($P<0.01$).

Discussion

DR is the leading cause of blindness in patients 20-70 years of age. The detailed mechanism of DR remains largely unknown. However, the breakdown of the inner endothelial BRB and subsequent retinal vascular leakage are the main causes of vision loss due to DR. A number of evidences have shown that early retinal microvascular damage due to diabetes mellitus is closely related to its immunoreactions/inflammation. There is accumulating evidence that DR is an inflammatory disease. Considering the present limitations of the development of new therapeutic strategies for DR treatment, it has become a necessity to focus on pharmacological treatment. In the present study, we demonstrated that: (1) FK506 can serve as a protector for the BRB in the early stage of diabetic rats; (2) FK506 can decrease the adhesion of leukocytes to endothelial cells in diabetic retinal vessels; (3) the effect of FK506 may be due to the inhibition of VEGF and ICAM-I levels in diabetic rats.

At the early stages of DR, no symptoms or mild symptoms appear. At the advanced stage, severe pathologies often lead to irreversible blindness if not properly treated [20]. Moreover, current therapeutic modalities, including vitrectomy [21] and laser photocoagulation [22], have been used to relieve symptoms. However, these therapeutic effects are not satisfactory. Therefore, there is a need for a novel and effective intervention approach at the early stage of DR that targets the initial injury and direct cause of the disease.

A previous study demonstrated that FK506 has an important anti-inflammatory property, which not only inhibits the release of pro-inflammatory mediators, but also inhibits the activation of leukocyte infiltration into the site of inflammation [16]. FK506 has been widely used to treat dry eye and superior limbic keratoconjunctivitis, which are ocular inflammatory diseases [23, 24]. We speculate that FK506 may exert its protective effects on the BRB. DR is characterized by retinal capillary circulatory disorders, which include the appearance of microaneurysms, increased vascular permeability, capillary occlusion and fibrous and neovascular proliferation. In vascular diseases, a crucial early phenomenon linked to diabetes mellitus was endothelial dysfunction. Since the BRB principally breaks down, vision loss in DR occurs. This leads to macular edema, retinal detachment and inner retinal and vitreous hemorrhage. In our study, the protective effects of FK506 on the BRB and blood vessels were clearly demonstrated.

Streptozotocin-induced diabetic rats were used because this animal model represents only cellular processes that have the characteristic of human non-proliferative DR. Experimental DR in streptozotocin-injected rodents has been described as an inflammatory disease, in which ICAM-1 plays a key role. Evidence revealed that the increase in ICAM-1 expression and leukostasis was correlated with the BRB breakdown [25]. Furthermore, BRB breakdown plays a vital role in many vascular diseases of the retina. It has been recognized that the major component of this functional barrier is at the level of the tight junctions between adjacent endothelial cells. In addition, maintaining the normal function of these junction-associated proteins in endothelial cells is essential to keep the normal function of the retina. If the expression of these proteins decreases, various vascular pathologies including diabetic retinopathy are caused. A number of studies have indicated that VEGF plays a significant role in this pathology [26, 27]. The administration of VEGF in retinal endothelial cells and the retina would decrease these tight junction-associated proteins, and subsequently increase leakage. Retinal vascular dysfunction caused by VEGF is also the major pathological change that occurs in DR. As the prominent mediator in the process of DR, the overexpression of VEGF has been believed to be correlated with vascular hyperpermeability and neovascularization in diabetic subjects [28, 29]. The effects of the dual-target intervention on the expression of VEGF and CTGF, as well as on the microvessel ultrastructure, have been examined in the retina of streptozocin-induced diabetic rats [30]. Therefore, we consider that the protective effects of FK506 against BRB breakdown might be associated to its modulation of VEGF levels in the retina. VEGF is overexpressed in diabetic rat retinas, and is involved in BRB leakage and leukocyte adhesion. However, we found that

VEGF content decreased in diabetic rats after FK506 treatment. More importantly, this observation was correlated with the decrease in BRB permeability. In present study, we have shown that FK506 inhibited leukocyte-endothelial cell interactions, and thereby suppressed BRB breakdown in diabetic retina. In addition, our experiments demonstrated that FK506 treatment suppressed the expression of ICAM-1, an important adhesion molecule that mediates leukocyte-endothelial cell interactions in diabetic retina. Eight days with diabetes significantly increased leukostasis within retinal vessels.

Diabetes also significantly increased the expression of VEGF. VEGF is another mediator that upregulates ICAM-1 expression on endothelial cells, and is an important cytokine in the induction of diabetic retinopathy [31]. FK506 significantly inhibited all of these abnormalities. Consistent with the reduction in ICAM-1, leukocyte accumulation was significantly reduced in advanced diseases. A number of studies on vascular endothelial cells have demonstrated that ICAM-1 is an important factor for the adhesion of leukocytes to the endothelium, and for the migration of leukocytes into tissues; which causes endothelial cell injury. This would result in increased retinal vascular permeability, retinal edema, and loss of visual acuity. ICAM-1 expression is upregulated in diabetic retinas, and causes leukocyte endothelial cell interactions [32, 33]. Therefore, the ability of FK506 to inhibit ICAM-1 expression demonstrates that FK506 can attenuate leukocyte-endothelial cell interactions, as well as subsequent endothelial damage and tissue injury. Indeed, the inhibition of leukocyte endothelial cell interactions by FK506 has been reported in many conditions. These results provide the best evidence to support the view that FK506 can inhibit leukocyte-endothelial cell interactions.

There are many limitations in present study. First, when compared to the chronic nature of diabetes, the duration of the administration of FK506 was rather short. In addition, quantitative measurements and studies on possible molecules by which FK506 might affect vascular permeability were not performed. Therefore, further studies are needed to elucidate the molecular and cellular mechanism underlying the protective effect of FK506 against diabetes-induced retinal vascular leakage.

Conclusions

In summary, the present study demonstrated that FK506 treatment protected the BRB from diabetic insults not only by decreasing VEGF and ICAM-1 expression, but also by maintaining the integrity of the BRB. Therefore, FK506 treatment may be an effective therapeutic approach for DR.

Disclosure of conflict of interest

None.

Authors' contribution

WZ carried out the study and drafted the manuscript. SL participated in the design of the study. JM conceived of the study, and participated in its design and helped to draft the manuscript. All authors read and approved the final manuscript.

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