Study on Vascular Endothelial Growth Factor and Its Receptor in the Vitreous of Diabetic Rats

Y Zhang[1], D Song[2], G Zhang[3]

ABSTRACT

Objective: To investigate the vitreous level of vascular endothelial growth factor (VEGF) and kinase insert domain-containing receptor (KDR) in diabetic rats, and to explore the role of VEGF and KDR in diabetic retinopathy.

Methods: Eighty-four adult Wistar rats were randomly divided into two groups. Fifty-eight rats in group A were injected intraperitoneally with streptozotocin to induce diabetes and 20 rats in group B were injected with physiological saline. Blood glucose meter was used to detect the blood glucose level at 72 hours after injection; blood glucose level ≥ 16.67 mmol/L was considered to be successful modelling. Blood glucose level was assayed and body mass was measured on the same modelling day, one week, two weeks and four weeks after modelling. Four weeks after modelling, the vitreous was taken and the VEGF and KDR levels were detected by enzyme-linked immunosorbent assay (ELISA). The eyeballs were fixed with paraform and embedded by petrolin for haematoxylin and eosin (H&E) staining.

Results: Forty-two rats survived and 16 rats died in group A. No rats died in group B. The blood glucose at one week, two weeks and four weeks between the two groups had statistical differences ($p < 0.05$). The weight at one week and two weeks between the two groups was not different but there was statistical difference at four weeks between the two groups ($p < 0.01$). The ELISA results showed that the VEGF and KDR levels were 0.276 ± 0.026 ng/mL and 2.936 ± 0.295 ng/mL in group A, 0.231 ± 0.021 ng/mL and 2.394 ± 0.227 ng/mL in group B, respectively. The VEGF and KDR levels of group A were higher than those of group B ($p < 0.05$).

Conclusions: The changes of VEGF and KDR levels in the vitreous of diabetic rats were related to the early retinopathy induced by diabetes.

Keywords: Diabetic rat models, kinase insert domain-containing receptor (KDR), vascular endothelial growth factor (VEGF), vitreous

Estudio del Factor de Crecimiento Endotelial Vascular y Su Receptor en el Humor Vítreo de Ratas Diabéticas

Y Zhang[1], D Song[2], G Zhang[3]

RESUMEN

Objetivo: Investigar el nivel vítreo del factor de crecimiento endotelial vascular (FCEV) y receptor con dominio inserto-quinasa (KDR) en ratas diabéticas, y explorar el papel de FCEV y KDR en la retinopatía diabética.

Métodos: Ochenta y cuatro ratas adultas Wistar fueron divididas aleatoriamente en dos grupos. A cincuenta y ocho ratas en el grupo A se les inyectó estreptozotocina por vía intraperitoneal para inducir diabetes, mientras que a 20 ratas en el grupo B se les inyectó una solución salina fisiológica. Se usó un medidor de glucosa en sangre para detectar el nivel de glucosa en sangre a las 72 horas después de la inyección. Un nivel de glucosa en sangre ≥ 16.67 mmol/L se consideró como un modelo exitoso. Se analizó el nivel de glucosa en sangre, y se midió la masa corporal en el mismo día del modelado, y una

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[1]Department of Ophthalmology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450000, Henan Province, China, [2]Jiaozuo Hospital of Traditional Chinese Medicine, Jiaozuo 454450, Henan Province, China and [3]Department of Biochemistry, Basic Medical College of Zhengzhou University, Zhengzhou 450000, Henan Province, China.

Correspondence: Dr Y Zhang, Department of Ophthalmology, The First Affiliated Hospital of Zhengzhou University, 1 Jianshe Road, Zhengzhou 450000, Henan Province, China. E-mail: youmeizhangcn@163.com

semana, dos semanas, y cuatro semanas después del modelado. Cuatro semanas después del modelado, se tomó el humor vítreo, y los niveles de FCEV y KDR fueron detectados mediante ensayo por inmunoabsorción ligado a enzimas (ELISA). Los globos oculares fueron fijados con para formaldehido e incrustados por petrolin para tinción (H & E) hematoxilina-eosina.

Resultados: *Cuarenta y dos ratas sobrevivieron y 16 ratas murieron en el grupo A. Ninguna de las ratas en el grupo B murió. La glucosa en la sangre a la semana, las dos semanas, y las cuatro semanas entre los dos grupos tuvo diferencias estadísticas (p < 0.05). El peso a la semana y a las dos semanas entre los dos grupos no fue diferente, pero hubo diferencia estadística a las cuatro semanas entre los dos grupos (p < 0.01). Los resultados de ELISA mostraron que los niveles de FCEV y KDR fueron 0.276 ± 0.026 ng/mL y 2.936 ± 0.295 ng/mL en el grupo A, 0.231 ± 0.021 ng/mL y 2.394 ± 0.227 ng/mL en el grupo B, respectivamente. Los niveles de FCEV y KDR del grupo A fueron superiores a los del grupo B (p < 0.05).*

Conclusiones: *Los cambios de nivel FCEV y KDR en el humor vítreo de las ratas diabéticas estaban asociados con la retinopatía temprana inducida por diabetes.*

Palabras claves: Modelos de diabetes en ratas, receptor con dominio inserto-quinasa (KDR), factor de crecimiento endotelial vascular (FCEV), humor vítreo

INTRODUCTION

Diabetes is a syndrome which is partly inherited but could also be triggered by immune dysfunction, microorganism infection, free radicals and trauma. These factors can induce the hypo-insulinism or insulin resistance, which leads to metabolic disorder of sugar, protein, fat, water and electrolytes. The classic symptoms of diabetes are loss of weight, polyuria (frequent urination), polydipsia (increased thirst) and polyphagia (increased hunger), all of which are caused by high blood sugar (1, 2).

Systemic microvascular lesions occur at an early stage of diabetes. Microvascular lesions are usually accompanied by abnormal microcirculation, which leads to complications of various organs. Microvascular lesions are mainly found in kidney glomerulus, fundus oculi, nerves and myocardium (3, 4).

Diabetic retinopathy (DR) is one of the most serious diabetic microvascular complications (5, 6). Previous studies found that vascular endothelial growth factor (VEGF) and its major receptor – KDR (kinase insert domain-containing receptor) – played a key role in DR. Moreover, the content and distribution of VEGF and KDR changed in the vitreous body and retina of diabetics (7–9). In this study, diabetes in a rat model was induced with streptozotocin, and VEGF and KDR were detected in the vitreous body of the rats to investigate the role of VEGF and KDR in DR.

SUBJECTS AND METHODS
Experiment animals

Eighty-four healthy adult Wistar rats with weight 200–250 g were purchased from Experiment Animal Center of Zhengzhou University. They were housed separately for adaptability for four days and weighed after being deprived of food for 12 hours. All rats were divided into two groups randomly – 64 rats in group A and 20 rats in group B. This study was carried out in strict adherence to the recommendations of the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. The protocol for ani-mal use has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the First Affiliated Hospital of Zhengzhou University.

Diabetes model

In order to induce a diabetic rat model, each rat was treated with intraperitoneal injections containing streptozotocin (STZ, 40 mg/kg; MP BIO, Santa Ana, CA, USA) once a day for four days. This was followed by assaying of concentrated blood glucose from the tail veins using blood glucose meter (Shanghai Roche Pharmaceutical Co., Shanghai, China). Fifty-eight rats were enrolled in group A with blood glucose concentration $\geq$ 16.67 mmol/L. In Group B, 20 rats were treated with intraperitoneal injections of physiological saline (9).

General state of health observation and preparation of retinal slices

Changes in appearance, weight and blood sugar in all the rats were recorded after one week, two weeks and four weeks of successful modelling. After four weeks, all the rats were killed and their eyeballs removed. Twenty-eight eyeballs of 14 rats from group A and 12 eyeballs of six rats from group B were stained with haematoxylin and eosin (H&E) after being fixed by 40 g/L paraformaldehyde and paraffin embedding.

Collection of vitreous bodies and ELISA assay

The eyeballs of the remaining rats were dissected under the microscope and two vitreous bodies from one of the rat's eyes were placed into sterile EP tubes. They were centrifuged at 3000 r/minute for 10 minutes and the

supernatant was kept in -70 °C freezer. Vascular endothelial growth factor and KDR enzyme-linked immunosorbent assay (ELISA) kits were purchased from Guangzhou Da An Gene Biotech Co. Microplate readers were from Beijing Zhongsan Jinqiao Biotech Co. Detailed steps of the operating instructions were followed.

Statistical analysis
Data analyses were carried out using SPSS version 14.0 (SPSS Inc, Chicago, IL, USA). All results were expressed as means ± S (X ± S). The data on the general state of health and blood glucose concentrations of the two groups of rats were compared using repeated measures of analysis of variance. The LSD (least significant difference) method was used to compare the concentrations of the vitreous VEGF and KDR of the two groups of rats. *P*-value less than 0.05 was considered statistically significant.

RESULTS
Effect on rats' general state of health
Fifty-eight diabetic rat models were successfully made in group A. After four weeks, 16 rats died while 42 survived. All rats in group B survived. Whereas rats in group A showed obvious symptoms of polydipsia, polyuria and polyphagia one week after modelling, the rats in group B did not. Four weeks after modelling, the rats in group A showed poor mental status and dull coat colour while the rats in group B were in good mental state and active with shiny coat colour. There was no statistical difference in weight between the two groups of rats at one and two weeks after modelling. However, four weeks after modelling, there were statistical

Table 1:   The changes of weight in the two groups of rats

Group/weight (g)	1 week	2 weeks	4 weeks
A	273.27 ± 32.16	285.76 ± 34.26	297.43 ± 33.50
B	269.85 ± 31.84	327.14 ± 38.98	374.57 ± 43.57*

*p < 0.05 *vs* group A

differences between the two groups of rats (t = 2.548, *p* = 0.041). The details are showed in Table 1.

Effect on rats' blood sugar
One week, two weeks and four weeks after modelling, there were statistical differences in blood sugar between the two groups of rats (t = 28.437, 27.143, 25.871, *p* < 0.01), an indication that diabetic rat models were successfully made in group A. The data are shown in Table 2.

Table 2:   The changes of blood sugar in the two groups of rats

Group/blood sugar (μmol/L)	1 week	2 weeks	4 weeks
A	27.64 ± 5.83	26.36 ± 5.12	25.29 ± 5.01
B	5.14 ± 1.76*	5.36 ± 1.73*	5.71 ± 1.86*

*p < 0.01 *vs* group A

Effect on rats' retina
Haematoxylin and eosin staining showed that the retinal cells were swollen and disorganized in group A. On the other hand, in group B, retinal cells had no obvious swelling and were well organized. Pathological changes of prophase DR were observed under the electron microscope and showed scattered microaneurysm, punctate bleeding or fragments in the retinal posterior pole, expansion of retinal veins and mild tortuosity.

Effect on the contents of VEGF and KDR
Twenty-eight eyeballs of 14 rats from group A and 12 eyeballs of six rats from group B were collected. The contents of VEGF and KDR in group A were much higher than those of group B as shown by the ELISA results in Table 3. There were statistical differences in blood sugar between the two groups (t = 2.317, 2.859, *p* = 0.048, 0.032).

Table 3:   Comparison of vascular endothelial growth factor (VEGF) and kinase insert domain-containing receptor (KDR) between the two groups of rats

Group	n	VEGF (ng/mL)	KDR
A	28	0.276 ± 0.026	2.936 ± 0.295
B	12	0.231 ± 0.021*	2.394 ± 0.227*

*p < 0.05 *vs* group A

DISCUSSION
At present, more than 90 million Chinese are diabetics, accounting for one-fifth of diabetic patients worldwide. Moreover, China ranks second in the whole world in terms of the prevalence of diabetes. Diabetes can lead to complications of the body organs, especially a series of complications in the eyes which can impact energy supply to those cells. In all complications, DR was the most common (10–12).

Diabetic retinopathy is divided into proliferative and non-proliferative. The non-proliferative phase is characterized by scattered microaneurysm, dot haemorrhage or fragments in the retinal posterior pole and expansion of retinal veins with mild tortuosity. As the disease progresses, the retina develops hard exudates and lint spots and diabetic patients complain of flashlight sensation and vision loss. In the proliferative phase of DR, vitreous haemorrhage and a wide range of neovascularization on the retinal and optic disc take place. Serious loss of vision occurs in the proliferative retinopathy patient. Stretching of the retina causes retinal detachment which leads to blindness in severe cases (13).

Vascular endothelial growth factor is a highly conserved dimeric glycoprotein which consists of two identical subunit (Mr 23000) synthesis with disulfide bond cross-links. There are four different variants of VEGF. The variations are due to differences in mRNA splicing, including VEGF121, VEGF165 and VEGF189 expression in human.

Vascular endothelial growth factor is widely expressed in the pathological process of tumour growth and ischaemic diseases. It works in vascular endothelial cells and only exists in these cells because of its specific receptors which are mainly regulated by an ischaemic and hypoxic environment (14, 15).

It is well known that DR belongs to ischaemic retinal diseases and diabetic patients have systemic changes in blood flow indicators: whole blood and plasma viscosity and fibrinogen which are significantly increased (16). Sustained high blood sugar and high blood viscosity make glycosylation end products accumulate in tissue protein for a long time, resulting in retinal ischaemia and hypoxia, retinal vascular atresia, avascular area formation and micro-arteriovenous 'short-circuit', eventually leading to the retinal venous flow stasis and emerging as a series of fundus lesions (17).

In this study, there were obvious differences in the general state of health and blood glucose levels of the two groups of rats over the various time periods (Tables 1–3). Vascular endothelial growth factor expression in the retina of DR rats was higher than in the control group and gradually increased with the duration of the study. Therefore, VEGF might be a risk factor for a series of lesions in DR. Zhou and Zhang believed that there was a correlation between the concentration of vitreous VEGF and severity of proliferative diabetic retinopathy (18). In our research, the VEGF of group A was higher than that of group B, indicating that VEGF secretion was significantly increased under the condition of retinal ischaemia and hypoxia. Vascular endothelial growth factor destroys tight junctions between endothelial cells, thereby causing the destruction of the blood-retinal barrier. The increasing concentrations of VEGF in group A promoted the early pathological changes of DR and increased its development.

Kinase insert domain-containing receptor is one of the important receptors for VEGF. Activation of KDR could cause vascular endothelial cell division, proliferation and migration (19). Previous studies have shown that KDR was mainly located in the retinal vascular leakage zone and associated with vascular leakage. After adding the KDR inhibitor, neovascularization of the vitreous and retina was inhibited, indicating that KDR played an important role in pathological retinal neovascularization (20). In this study, the content of KDR in group A was higher than that of group B. Thus, we speculated that the increased content of KDR in the vitreous of diabetic rats promoted diabetic retinal vascular leakage, which affected the development of DR.

In the present study, vitreous concentrations of VEGF and KDR were higher in diabetic rats than in the control group, which means that VEGF and KDR played an important role in vascular leakage and neovascularization at prophase of DR. In subsequent studies, we may inhibit the diabetic retinopathy angiogenesis by affecting VEGF and KDR, thus achieving the purpose of treatment of diabetic retinopathy and possibly providing a new way to treat DR.

This study has some limitations due to the shortness of the observation time and the use of a diabetic rat model instead of actual diabetic patients. In order to study the relationship between VEGF and KDR and neovascularization in DR in future research, diabetic patients would be used and the observation would be extended.

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