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· Original article ·

Streptozotocin induced diabetic retinopathy in C57 mice and the expression of some pro-angiogenic molecules

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链脲佐菌素诱导的小鼠糖尿病视网膜病模型及促血管新生分子的表达

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摘要

目的:建立链脲佐菌素(streptozotocin, STZ)诱导的小鼠增殖性糖尿病视网膜病(proliferative diabetic retinopathy, PDR)动物模型,并观察在增殖性糖尿病视网膜病发生、发展过程中血管内皮生长因子(vascular endothelial growth factor, VEGF)及其受体(VEGFR1, VEGFR2),金属基质蛋白酶(matrix metalloproteinase, MMP)2, MMP9 表达的变化。

方法:C57BL/6J 小鼠连续 5d 腹腔注射 STZ (55 mg/kg)。末次注射后 7d 检测血糖浓度。糖尿病诱导成功的小鼠和正常小鼠继续饲养 3~5mo。实验结束后进行视网膜病理组织观察,并利用 CD31 免疫荧光染色检测视网膜血管的分布情况。采用实时定量荧光 PCR 分析检测 VEGF, VEGFR1, VEGFR2, MMP2, MMP9 的基因表达。

结果:视网膜组织病理观察和 CD31 免疫荧光染色实验结果均表明 5 月龄的糖尿病小鼠视网膜组织中血管数目明显比同月龄正常小鼠多。同时,与同月龄正常小鼠相比,5 月龄糖尿病小鼠视网膜组织中 VEGF, VEGFR1, VEGFR2, MMP2, MMP9 的基因表达也明显增加。

结论:本研究表明 STZ 诱导的糖尿病小鼠在 5 月龄时发生了增殖性糖尿病视网膜病的病变,期间视网膜组织中 VEGF, VEGFR1, VEGFR2, MMP2, MMP9 的基因表达都明显增加。

关键词:糖尿病视网膜病变;VEGF;VEGFR1;VEGFR2;MMP2;MMP9

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Abstract

• AIM: To establish the mice model of streptozotocin (STZ)-induced proliferative diabetic retinopathy (PDR), and observe the altered expression of some pro-angiogenic molecules such as vascular endothelial growth factor (VEGF) and its receptors (VEGFR1 and VEGFR2), and matrix metalloproteinase (MMP2 and MMP9) during the development of PDR.

• METHODS: C57BL/6J mice were intraperitoneal injected with STZ (55 mg/kg) for 5 consecutive days, and blood glucose concentrations were measured after 7d of the injection. The diabetic mice were further housed for 3, 4, 5mo respectively after the development of diabetes. Histological evaluation of retinas was performed. The retinal vessels were detected by immunofluorescence staining with the cluster of differentiation 31 (CD31). The mRNA expression of VEGF, VEGFR1, VEGFR2, MMP2 and MMP9 in mice retinas was detected by Real-time PCR analysis.

• RESULTS: Retinal histological observation and CD31 staining both demonstrate that there are more vessels in diabetic mice than in normal control mice at 5mo after the development of diabetes. As compared with normal control, the mRNA expression of VEGF, VEGFR1, VEGFR2, MMP2 and MMP9 are all increased in diabetic mice at 5mo after the development of diabetes.

• CONCLUSION: This study demonstrates that PDR is occurred at 5mo after the development of diabetes in STZ-induced diabetic mice. In addition, the mRNA expression of VEGF, VEGFR1, VEGFR2, MMP2 and MMP9 are all increased after the development of PDR.

• KEYWORDS: diabetic retinopathy; VEGF; VEGFR1; VEGFR2; MMP2; MMP9

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INTRODUCTION

Diabetic retinopathy (DR) is one of the most common microvascular complications of diabetes[1]. It is reported that after 10y with the development of diabetes, nearly 60% of diabetic patients will progress to proliferative DR (PDR), and about 35% of diabetic patients are reported progression to severe vision loss[2]. DR seriously decreases the quality of life in diabetic patients, and it also brings heavy economic burden to diabetic patients and country. According to the development of DR, it has two distinct phases: an early non-proliferative phase (NPDR) characterized by increased vascular permeability and intra-retinal hemorrhage; and a late proliferative phase (PDR) characterized by retinal neovascularization[3-4]. During the process of NPDR, hyperglycemia-induced damage in the retina are associated with the loss of retinal capillary pericytes, thickening of the vascular layers, and breakdown of the blood retinal barrier, which will lead to retinal ischemia and hypoxia. Proliferative growth of new vessels and subsequent tractional retinal detachment will occur when NPDR proceeds to PDR[3,5], and thus it finally lead to severe vision loss.

Generally, numerous pro-angiogenic growth factors are involved in the regulation of retinal neovascularization during the process of PDR. Among which, vascular endothelial growth factor (VEGF) is considered the most important pro-angiogenic growth factor[6]. VEGF exerts pro-angiogenic function mainly *via* binding to its two tyrosine kinase receptors: VEGFR1 (Flt-1) and VEGFR2 (KDR/Flk-1)[7-8]. In addition, extracellular matrix degradation is another key event in the process of neovascularization. Matrix metalloproteinases (MMPs) are the important extracellular matrix-degrading enzymes[9]. Despite a lot of studies on the mice model of DR, most of them focus on NPDR, whereas there is no much report on PDR[10]. In this study, we observed the development of PDR in STZ-induced diabetic mice, and the expression of some pro-angiogenic molecules such as VEGF, VEGFR1, VEGFR2, MMP2 and MMP9.

MATERIALS AND METHODS

Reagents The antibody for the cluster of differentiation 31 (CD31) and fluorescein isothiocyanate (FITC)-conjugated anti-rat IgG were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Trizol reagent was purchased from Life Technology (Carlsbad, CA, USA). PrimeScript RT Master Mix and SYBR Premix Ex TaqTM were purchased from Takara (Shiga, Japan). Streptozotocin (STZ) and other reagents unless indicated were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Animals Fifty C57BL/6J healthy male mice (18-22 g, 6wk) were purchased from the Shanghai laboratory animal center of Chinese Academy of Sciences (Shanghai, China). The animals were maintained under controlled temperature (23±2℃), humidity (50%), and 12-hour light/dark cycle. All animals were allowed free access to food and water. All experimental protocols conducted were performed in accordance with the institutional animal care guidelines approved by the Experimental Animal Ethical Committee of Shanghai University of Traditional Chinese Medicine.

Mice Model of Diabetic Retinopathy Twenty-eight mice received an intraperitoneal injection of 55 mg/kg STZ for 5 consecutive days. STZ was dissolved in sodium citrate buffer (0.1 mol/L, pH 4.2). The other twenty-two mice in the control group received injection of an equal volume of physiological saline, and those mice were randomly divided into three groups. Blood glucose concentrations were measured 7d after the injection. Animals with blood glucose concentrations higher than 16.5 mmol/L were considered as diabetic mice. Twenty-seven animals were considered diabetic and divided randomly into three groups of nine mice. At 3, 4 and 5mo after the injection of STZ, respectively, the mice were anesthetized by sodium pentobarbital (40 mg/kg, i.p.), blood sample were taken from the abdominal aorta, and the eyeballs were removed immediately. In each group, there were 5 eyeballs used for retinal immunofluorescence staining, 4 eyeballs were used for histological assessment, and the remaining 5-9 eyeballs were used for Real-time PCR analysis.

Serum Glucose Concentration Analysis Fresh blood was obtained from mice of all groups and put at room temperature for 60min to clot. Serum was isolated following the centrifugation at 840 g for 15min. Serum glucose concentration was detected by an automatic biochemical analyzer (Hitachi, Japan).

Retinal Immunofluorescence Staining The eyeballs were fixed with 4% paraformaldehyde solution overnight at 4℃ after removed from the mice. The cornea was dissected with a circumferential limbal incision, after removal of the lens and vitreous, retinas were carefully dissected from the eyeballs under a microscope (SZX7, Olympus, Japan). Isolated retinas were incubated with blocking buffer (5% BSA, 0.5% Triton X-100 in PBS) for 2h at room temperature, and then were incubated with CD31 antibody which were diluted in solution beffer (1% BSA, 0.5% Triton X-100 in PBS) for 2d at 4℃. After washing 6 times, the retinas were incubated with FITC-conjugated anti-rat IgG antibody for 2h. After washing 6 times again, the retinas were placed on a slide glass, mounted in gelatin, covered with a cover slip, and pictured under the fluorescence microscope (IX81, Olympus, Japan). The quantitative of the vessels was counted as previously described method[11-12].

Histological Assessment of Retinas Retinas were carefully dissected and embedded in paraffin. Retinal tissues were sectioned (5 μm) crossed the optic nerve, stained with haematoxylin and eosin, and regions near the optic nerve were observed under the microscope (H500S, Nikon, Japan).

Real-time Polymerase Chain Reaction Analysis Total mRNA was extracted from retinas by using Trizol reagent according to the manufacturer's protocol. The single strand cDNA was synthesized according to the manufacturer's protocol. Real-time PCR was Performed using a SYBR green premix kit. Relative expressions of target genes were normalized

Table 1 **Sequences of primers used for Real-time PCR**

Target	Primer	Sequence
VEGF	FP	5'-GCTACTGCCGTCCGATTGAG-3'
	RP	5'-ACTCCAGGGCTTCATCGTTACAG-3'
VEGFR1	FP	5'-CCTGATGGGCAAAGAATAACAT-3'
	RP	5'-ATTTGGACATCTAGGATTGTATTGG-3'
VEGFR2	FP	5'-GTGGTAAGTTGCGATTGTTGTG-3'
	RP	5'-TGAACATTCGCCTTCTTTGATA-3'
MMP2	FP	5'-AACATGTACAGGGTCGGAGACT-3'
	RP	5'-CATTCCCGTTGGCTGTCT-3'
MMP9	FP	5'-GGTACAGCCTGTTCCTGGTGG-3'
	RP	5'-ATGCCGTCTATGTCGTCTTTATTCA-3'
Actin	FP	5'-TTCGTTGCCGGTCCACACCC-3'
	RP	5'-GCTTTGCACATGCCGGAGCC-3'

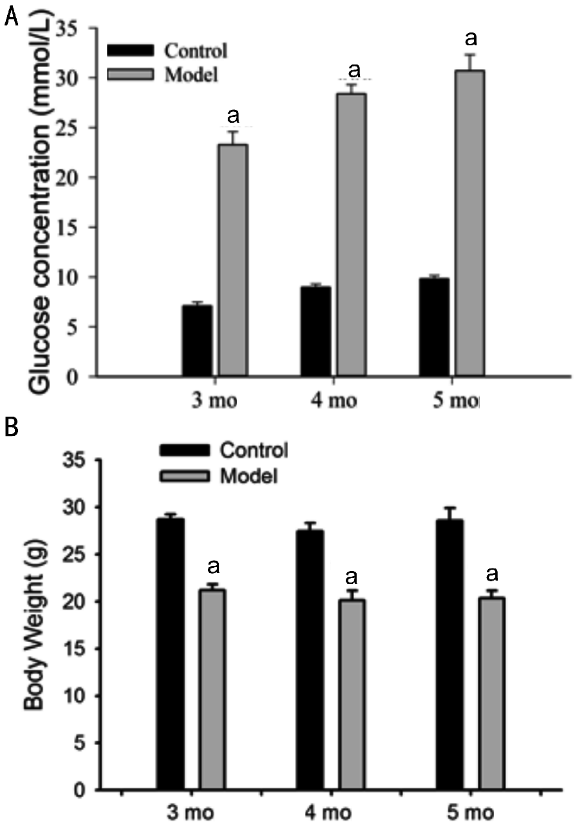

Figure 1 Analysis of serum glucose and body weight A: Serum glucose; B: Body weight. $n = 6 - 10$. ${}^{a}P < 0.001$ *vs* control mice at the same age.

to actin, analyzed by $2^{-\Delta\Delta Ct}$ method and given as ratio compared to control. The primer sequences used in this study are shown in Table 1.

Statistical Analysis Data were expressed as means ± standard error of the mean (SEM). The significance of differences between groups was evaluated by one-way ANOVA with LSD post hoc test; and $P < 0.05$ was considered as statistically significant differences.

RESULTS

Glycemia and Body Weight As shown in Figure 1A, serum levels of glucose in STZ-treated mice were all higher than 16.5 mmol/L at 3, 4, 5mo after the development of diabetes. Whereas serum glucose levels in normal mice were all lower than 16.5 mmol/L at different month. In addition, serum levels of glucose in STZ-treated mice were obviously higher than in normal control mice ($P<0.001$) at the same month. Furthermore, the body weight (Figure 1B) in mice at 3, 4, 5mo after the development of diabetes was lower than in normal mice at the same month ($P<0.001$).

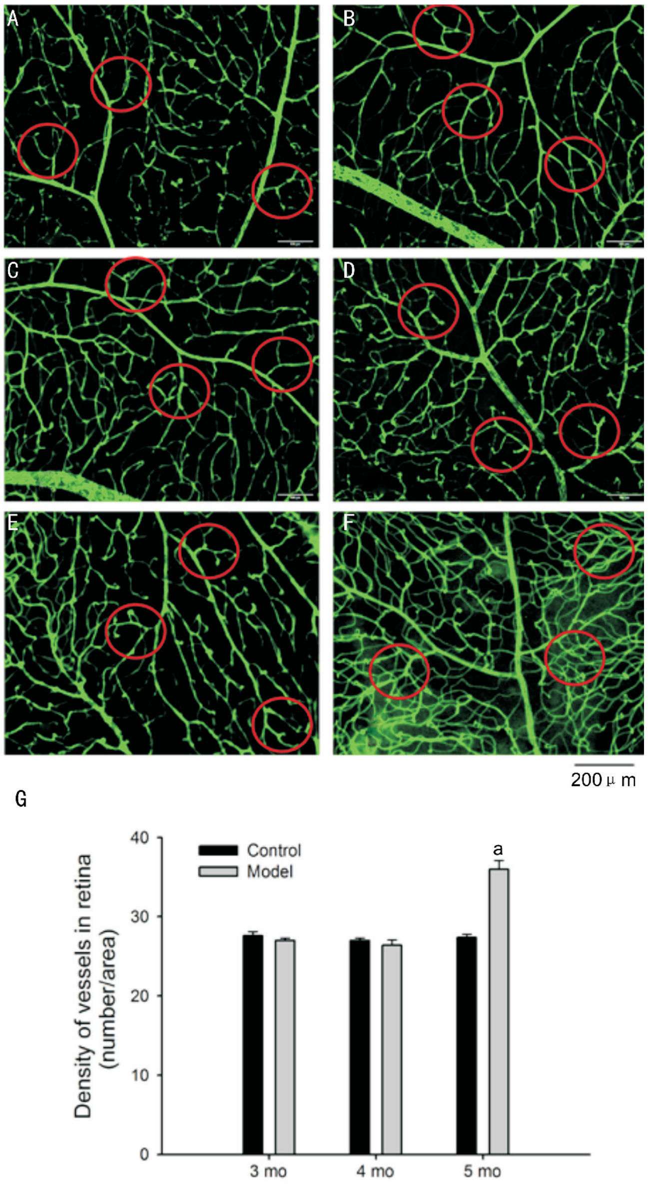

Figure 2 Immunofluorescence staining of retinal vessels in normal and diabetic mice A: Normal mice of 3mo; B: STZ-induced diabetic mice of 3mo; C: Normal mice of 4mo; D: STZ-induced diabetic mice of 4mo; E: Normal mice of 5mo; F: STZ-induced diabetic mice of 5mo; G: Quantitative results of retinal vessels. The scale in the pictures represents 200 μm. $n = 4$. ${}^{a}P < 0.05$ *vs* control mice at the same age.

Immunofluorescence staining of retinal vessels retinal vessels were stained with CD31, which is generally used to identify endothelial cells. The portion marked with a circle showed the larger vessels with small vessels branch off. As shown in Figure 2A-2D, there was no much difference in retinal vessels between normal and STZ-treated mice at 3 and 4mo after the development of diabetes. However, the small vessels

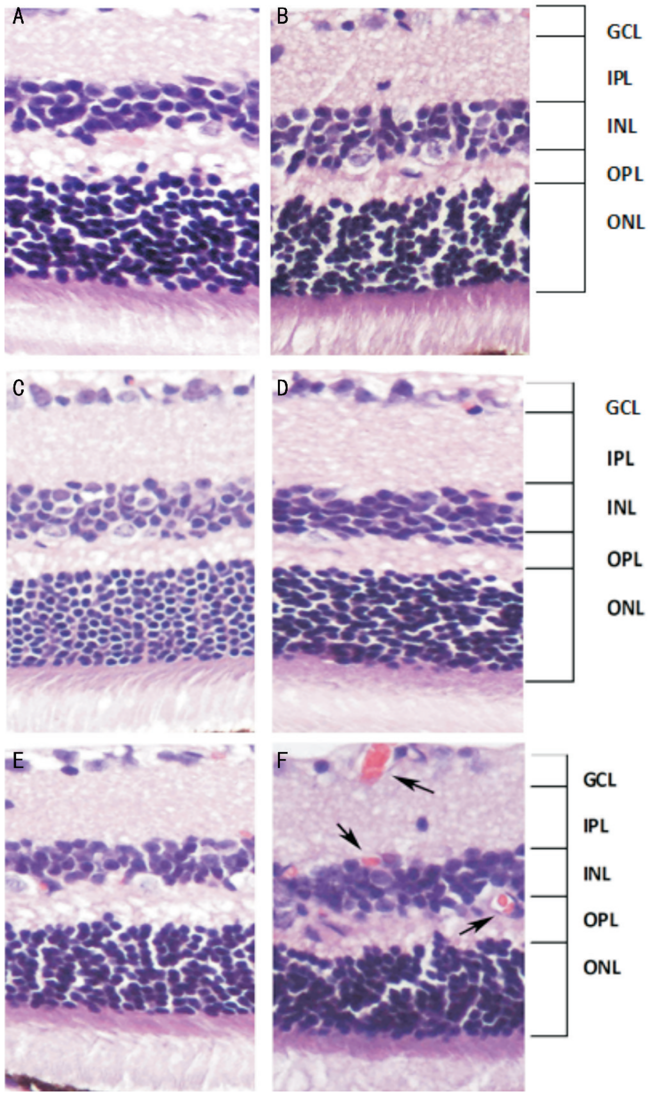

Figure 3　Histological evaluation of retinas　A: Normal mice of 3mo; B: STZ-induced diabetic mice of 3mo; C: Normal mice of 4mo; D: STZ-induced diabetic mice of 4mo; E: Normal mice of 5mo; F: STZ-induced diabetic mice of 5mo (×200). GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer. Arrows point out the retinal vessels. The representative pictures are from 4 mice.

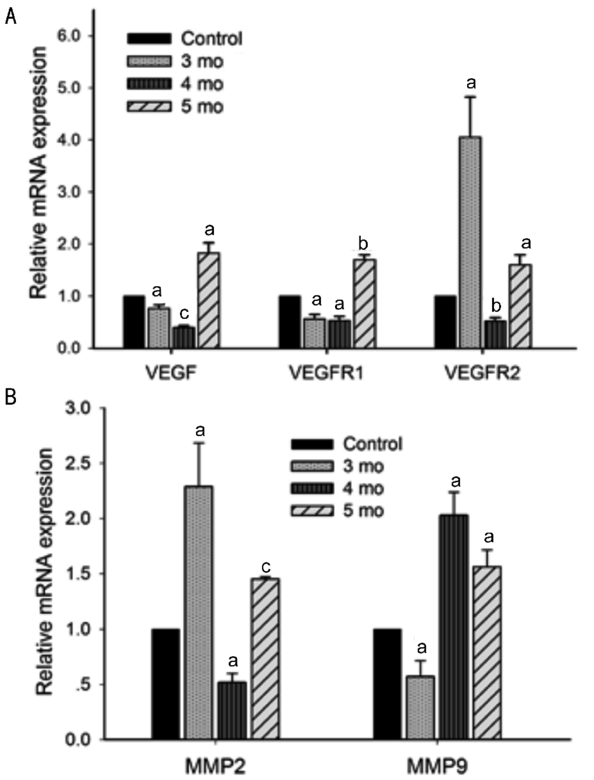

Figure 4　The mRNA expression of VEGF, VEGFR1, VEGFR2, MMP2 and MMP9　A: The mRNA expression of VEGF, VEGFR1 and VEGFR2; B: The mRNA expression of MMP2 and MMP9. $n=6$. The reaction was carried out in triplicate samples for at least 3 separate assays. $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$ *vs* control mice at the same age.

branch off number and length were increased and rearranged in STZ-treated mice at 5mo after the development of diabetes compared with the normal mice (Figure 2E and 2F). In addition, quantitative of the retina vessels showed that the density of vessels in STZ-treated mice retinas at 5mo after the development of diabetes was increased as compared with the normal control mice (Figure 2G) ($P<0.01$).

Histological Assessment　The whole retina from central to peripheral area was observed. There were no significant changes of the overall retinal thickness and the thickness of each layers in retinas at both 3 and 4mo after the development of diabetes compared with the normal control mice (Figure 3A-3D). However, at 5mo after the development of diabetes, as the arrows point out, there were more vessels even vascular cluster appeared in the ganglion cell layer (GCL) and inner nuclear layer (INL) in diabetic mice than in normal control mice (Figure 3E and 3F), which reflecting a pathological retinal angiogenesis process. As indicated by arrows, about 4 vessels per picture were found in retinas of mice at both 3 and 4mo after the development of diabetes. However, the vessel number was increased to about 7 in retinas of mice of at 5mo after the development of diabetes.

Expression of VEGF, VEGFR1, VEGFR2, MMP2, and MMP9 in Retinas　The retinal mRNA expressions of VEGF, VEGFR1, and VEGFR2 in mice at 3, 4 and 5mo after the development of diabetes were detected. At 3mo after the development of diabetes, the mRNA expressions of VEGF and VEGFR1 were both decreased, but VEGFR2 expression was increased in diabetic mice ($P<0.05$; Figure 4A). At 4mo after the development of diabetes, the mRNA expressions of VEGF, VEGFR1, and VEGFR2 were all decreased in diabetic mice ($P<0.05$, $P<0.01$ and $P<0.001$ respectively; Figure 4A). Furthermore, at 5mo after the development of diabetes, the mRNA expressions of VEGF, VEGFR1, and VEGFR2 were all increased in diabetic mice ($P<0.05$, $P<0.01$ respectively; Figure 4A).

We detected the mRNA expression of MMP2 and MMP9 in mice retinas. Our results showed that retinal MMP2 expression was increased but MMP9 was decreased in diabetic mice at 3mo after the development of diabetes ($P<0.05$; Figure 4B). At 4mo after the development of diabetes, retinal

MMP2 expression was decreased but MMP9 expression was increased in diabetic mice ($P < 0.05$; Figure 4B). Furthermore, at 5mo after the development of diabetes, the retinal mRNA expression of both MMP2 and MMP9 was increased in diabetic mice ($P<0.05$, $P<0.001$; Figure 4B).

DISCUSSION

DR has becomethe main cause of blindness in adults in the world[13]. Great efforts have been paid to the research on DR, and some animal models of DR have been developed, but most of them showed the early characteristics of DR and just little of them showed the late proliferative stage of DR. In addition, different labs arrive at different results even with the same animal model[10]. There was report that retinal neovascularization was occurred in STZ-induced diabetic mice after 17wk of hyperglycemia[14]. However, in another report, the presence of neovascularization were appeared in the Ins2 (Akita) mice, which is a genetic model of type 1 diabetes, after 8 to 9mo of hyperglycemia[15]. Thus, the concrete time of the occurrence of neovascularization in diabetic mice is not yet conclusive. From the results of retinal vessels staining in this study, we found that retinal neovascularization was occurred in STZ-induced diabetic mice at 5mo after the development of diabetes. Histological evaluation of retina also demonstrates that there were more vessels in STZ-induced diabetic mice at 5mo after the development of diabetes.

VEGF is the major pro-angiogenic growth factor, and it exerts pro-angiogenic activity *via* binding with its receptors such as VEGFR1 and VEGFR2[16-17]. It is well-known that VEGF/VEGFR2 signaling axis plays important roles in angiogenesis-related diseases such as tumor, DR, *etc*[18-20]. In addition, intravitreal injection of anti-angiogenic drugs especially VEGF inhibitors is considered as a feasible treatment of DR recently[21-22]. Numerous reports have shown the increased VEGF expression in patients with DR[23-25]. There are strong evidences about the increased VEGF, VEGFR2 expression in STZ-induced diabetic rats, but how long the VEGF and VEGFR2 expression is increased after the development of diabetes is not very consistent in different reports[12,26-28]. In addition, there is no much study on VEGF and VEGFR2 expression in STZ-induced diabetic mice. Our present study demonstrated that the increased mRNA expression of VEGF and VEGFR2 was appeared in STZ-induced diabetic mice at 5mo after the development of diabetes, which is consistent with the occurrence of retinal neovascularization. These results indicate the important role of VEGF/VEGFR2 in initiating retinal angiogenesis in STZ-induced diabetic mice at 5mo after the development of diabetes. There is a report that VEGF is decreased during the progressive of DR[29], which may contribute to the explanation for the decreased mRNA expression of VEGF in STZ-induced diabetic mice at 3 and 4mo after the development of diabetes in this study. Meanwhile, we also found that the mRNA expression of VEGFR2 was evidently increased at 3 and 5mo, but decreased at 4mo after the development of diabetes; which may be related with the progression of NPDR into PDR, however the concrete reason to cause such phenomena needs further investigation. Previous studies have showed that VEGFR1 was decreased or did not change in STZ-induced DR in mice[27-28]. In our study, we found that VEGFR1 expression was decreased in STZ-induced diabetic mice at 3 and 4mo after the development of diabetes, which is consistent with the previous report[27]. Furthermore, our results showed that the mRNA expression of VEGFR1 was increased in STZ-induced diabetic mice at 5mo after the development of diabetes, which may also contribute to the retinal neovascularization in STZ-induced diabetic mice at 5mo after the development of diabetes.

MMPs is a family of proteinases, which degrade at least one component of the extracellular matrix (ECM). MMPs has emerged as an important regulator in many normal and pathological processes, such as organ development, tissue remodeling, inflammation and angiogensis[30-31]. In addition, there are reports that the expression and bioavailability of VEGF can be modulated by MMPs, the degradation of ECM by MMPs will further facilitate the secretion of VEGF, and subsequent endothelial cell migration and tube formation[32-33]. Recent studies have shown that MMP2 and MMP9 expression was increased in the development of DR[34-35]. In addition, there is report that MMPs may have dual roles in the development of DR: MMP2 and MMP9 facilitate the apoptosis of retinal capillary cells in the stage of NPDR, while they also facilitate retinal neovascularization in the stage of PDR[36]. Our results showed that the mRNA expression of MMP2 was increased in diabetic mice at 3 and 5mo after the development of diabetes, and the mRNA expression of MMP9 was increased in diabetic mice at 4 and 5mo after the development of diabetes. The increased expression of MMP2 and MMP9 may also contribute to the development of PDR.

In summary, the animal model of STZ-induced PDR in C57BL/6J mice was successfully established in our study, which will be helpful for the further study on PDR using this mice model. In addition, this study showed that the mRNA expressions of VEGF, VEGFR1, VEGFR2, MMP2 and MMP9 were all increased in diabetic mice during the development of PDR, which further evidenced the important roles of those signals in regulating the development of PDR.

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