The Change of Retinal Microvascular, Microstructure and Expression of IL-6, CD18, ICAM, TNF-α and VEGF in The Early Stage of Rat Diabetic Retinopathy

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Research Article

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Abstract

Background: Diabetic retinopathy (DR) is a leading cause of vision loss and blindness. The purpose of this project is to deeply observe the change of retinal microvascular, microstructure and expression of IL-6, CD18, ICAM, TNF-α and VEGF at the early stage of DR in rats with streptozotocin-induced diabetes mellitus (DM).

Methods: The fluorescein fundus angiography was used to examine fundus of living organisms, retinas were obtained for hematoxylin and eosin staining, periodic acid-Schiff staining, fluorescence imaging techniques and quantitative real-time PCR, while vitreous humors were isolated for vascular endothelial growth factor (VEGF)-A ELISA in diabetes group (n=25) and normal group (n=25) at 8th day, 4th week, 6th week, 8th week and 10th week after the onset of DM.

Results: In this study, we observed not only the decrease of RGCs and the increase of E/P ratio, acellular capillaries and type IV collagen-positive strands began to occur on 8th day after induction, but the vascular permeability and neovascularization buds began to happen in diabetes group in 8th week, while the expression of VEGF-A, VEGF mRNA, IL-6 mRNA, ICAM mRNA and TNF-α mRNA began significantly higher in diabetes group compared with normal group (P<0.01) on 8th day after induction and remained high expression level throughout the 10-week observation period. However, the expression of CD18 RNA began significantly higher in 4th week after induction and reached peak in 6th week.

Conclusions: In conclusion, the retinal microvascular injury, ganglion cell changes and high expression of VEGF-A, VEGF mRNA, IL-6 mRNA, ICAM mRNA, TNF-α mRNA and CD18 mRNA were happened on very early stage. The results offer new insight into the pathogenesis of diabetic retinopathy, and provide novel targets to inhibit the ocular disease.

Background

Diabetic retinopathy (DR) remains a leading cause of vision loss in the working age population of industrialised regions[1]. A third of the 463 million people with diabetes[2] have signs of diabetic retinopathy, and a third of these might suffer from severe retinopathy or macular oedema[3]. Loss of pericytes and RGCs, VEGF overexpression, with compensatory synthesis and deposition of extracellular proteins, characterizes early diabetic retinopathy. In addition, a variety of inflammatory mediators on the retina can be up-regulated in the early stage of diabetes, including ICAM-1, VEGF, NF-kB, iNOS and TNF-α, and local inflammatory response plays an important role in the occurrence and development of DR [4, 5].

For the early pathological changes, occurrence and development of DR, researchers have carried out some studies. One study[6] indicated that retinal ganglion cells (RGCs) apoptosis increased during the 4th week of the diabetes course, and this early abnormality of neurons may be due to the loss of nerve cells, Moreover, Kuwabara T et al[7] found that microvascular pericytes were lost in the early stage of diabetic retinal tissue. Loss of pericytes in the vascular wall leads to the formation of microhemangioma. Ayalasomayajula et al[8] found that the vitreous VEGF expression of diabetic rats was significantly higher
than that of normal control group on the 8th day after induction. In the 4-week observation period, VEGF expression in vitreous of diabetic rats was the highest at the 4th week, which was significantly higher than that of normal control group[9]. Overexpression of VEGF will transform or alter the occurrence of neovascularization and improve vascular permeability, leading to retinal structural and functional abnormalities [10].

Although the above studies have studied the pathological changes of retinal structure in the early stage of DR, they also urge us to understand the pathological changes of retinal structure in the early stage of DR at different time nodes, so as to provide an important theoretical basis for the early intervention of DR. This study was to conduct in-depth observation and study on the occurrence and development of the early stage of DR in diabetic rats, especially using fluorescein fundus angiography (FFA) for examining fundus of living organisms, as to further clarify the retinal microvascular injury, microstructure changes and expression of IL-6, CD18, ICAM, TNF-α and VEGF in the early stage of DR (Fig. 1).

1. Material And Methods

1.1. Animals, diabetes induction and experimental groups.

A total of 50 8–9 week-old male Sprague-Dawley rats were purchased from the Animal Center of Nanchang University (Nanchang, China). All experiments were conducted in accordance with the Instruction and Administration of Experimental Animals, as well as the ARRIVE guidelines, and were approved by the First Affiliated Hospital of Nanchang University. DM was induced by a single intraperitoneal injection of 60 mg/kg body weight streptozotocin(STZ) (Sigma-Aldrich; Merck Millipore). A total of 25 diabetic rats were treated as diabetes group and 25 normal rats without any treatment were used as the normal group. The rats in each group were sacrificed after FFA examination on the 8th day, 4th week, 6th week, 8th week and 10th week after induction, respectively.

1.2. Animals examination of fluorescein fundus angiography (FFA).

Fluorescein Fundus angiography (FFA) is one of the common and main methods for diagnosis of DR. The rats in each group were treated with FFA (Heidelberg Spectraalis HRA, Heidelberg, Germany) examined the right eye of rats. During FFA examination, SD rats were intraperitoneally injected with 10% sodium fluorescein injection (0.001 mL /g, International Medication Systems, Dunstable, United Kingdom) for quick examination.

1.3. H&E-stained retinal and periodic Acid-Schiff stain (PAS)-stained preparations.

One-fourth of the retinal tissues were subsequently sectioned (5 µm), stained with H&E and examined under a light microscope (magnification, x400; Zeiss AG, Oberkochen, Germany) to determine the number and area of RGCs present in the sample. One-fourth of retinal tissues were stained with PAS and examined under a light microscope (magnification, x400; Zeiss AG) to calculate the ratio of endotheliocytes to pericytes (E/P) and the number of acellular strands.
1.4. Estimation of VEGF-A in vitreous humor.

Levels of VEGF-A protein in the vitreous homogenates were estimated using a rat VEGF-A ELISA kit capable of detecting both VEGF-A isoforms (RayBiotech Inc, Norcross, GA, USA), according to the manufacturer's instructions. The antibodies in the kit have > 95% cross-reactivity with rat.

1.5. Fluorescence imaging techniques for flat retinal preparations.

Retinal flat-mounts were processed to visualize the vascular basement membrane by immersing them in marker solutions. Prior to immersion staining, the retinal flat-mounts were incubated at room temperature. Subsequently, the flat-mounts were immersed overnight in a marker solution containing rabbit polyclonal anti-type IV collagen antibody solution (1:300; ab19808; Abcam, Cambridge, UK) for basement membrane [9]. Fluorescent goat anti-rabbit immunoglobulin IgG (1:45; BA1105; Wuhan Boster Biological Technology, Ltd, Wuhan, China) was used as a secondary antibody. Subsequent the retinal flat-mounts were placed into DAPI. The retinal flat-mounts were then mounted on a Vectashield (Wuhan Boster Biological Technology, Ltd.) and analyzed using a Zeiss LSM 710 confocal laser scanning microscope to determine the number of type-IV collagen strands and the area and number of retinal neurocyte.

1.6. Quantitative Real-time PCR Analyses for VEGF and Various Inflammation-Related Molecules of Retina.

In order to measure the mRNA expression levels of VEGF, IL-6, CD18, ICAM and TNF in retinal tissue, total RNA was isolated from the one quarter of the retinal tissue remains using an extraction reagent (TRIzol; Invitrogen, Carlsbad, CA) and reverse-transcribed with a HiFiScript cDNA Synthesis Kit (First-Strand, CoWin Biosciences, China). PCR was performed using TaqDNA polymerase (Servicebio®, WuHan, China) in a thermal controller (Gene Amp PCR system; Applied Biosystems, Foster, CA).

1.7. Image processing and statistical analysis.

IPP 6.0 and ImageJ 2.0 were used to process images, and IBM SPSS 19.0 statistical software was used for statistical analysis of the obtained data. One-way ANOVA was conducted for multiple mean values, and independent sample t-test was conducted for data between groups. All data were treated with mean ± standard error. P < 0.05 was statistically significant.

2. Results

2.1. Animals examination of FFA

The optic disc of SD rats was located in the center of the retina and the retinal blood vessels were radiated (Fig2). After intraperitoneal injection of sodium fluorescein in SD rats for 3-5s, retinal arteries began to fill, retinal vein laminar flow was observed for 5-7s, and retinal vessel fluorescence decreased significantly for 3-6min, until the fluorescence disappeared completely. The whole skin of SD rats was
yellow at the end of examination. During the 10 weeks observation period after induction of DM, the SD rats in the diabetes group showed obvious vascular tortuosity and dilation at the 6th week, and the peripheral roughness and leakage began to appear at the 8th week, while the obvious vascular leakage and dilation appeared at the 10th week. However, no such phenomenon was observed in the normal group during the observation period of 10 weeks after induction.

2.2. Retinal H&E staining.

The number of RGCs of SD rats in the diabetes group decreased significantly compared with that in the normal group (P < 0.01) on the 8th day after induction, and with the progression of disease, the number of RGCs in the diabetes group decreased gradually. However, there was no significant difference in the number of RGCs in the diabetes group at 8th week and 10th week (P > 0.05), and the number of RGCs tended to be stable. Furthermore, there was no significant difference in retinal ganglion cell area (P > 0.05) between the diabetes group and the normal group during the observation period of 10 weeks after induction (P > 0.05). We also found that microvascular dilatation was observed in 6th week, the formation of neovascularization bud and obviously microvascular dilatation were observed in 8th week, while typical neovascularization bud was observed between the ganglion cell layer and the inner nuclear layers at the 10th week. (Fig3)

2.3. Retinal PAS staining.

The E/P ratio in retinal tissue of SD rats in the diabetes group began to be significantly higher than that in the normal group at the 4th week (P < 0.01), and with the progress of disease, the diabetes group and the normal group always maintained a significant difference. We also found that the number of acellular capillaries was increased on the 8th day after induction in the diabetes group, and the trend of the increase was worse with the progress of the disease course. The significant difference began to appear on the 4th week after induction of DM (P < 0.01). At 8th week, neovascularization buds and a large number of acellular strands were observed, while typical neovascularization buds and a large number of acellular filaments were also observed at 10th week. (Fig4)

2.4. Estimation of VEGF-A in vitreous humor

On the 8th day after induction of DM, the concentration of VEGF-A in vitreous cavity of SD rats in diabetes group began to be higher than that in normal group at the same time point (P < 0.01), and remained at a high concentration with the progression of disease. However, VEGF-A concentration in vitreous cavity of SD rats in diabetes group began to increase further at the 6th week after the successful modeling, and reached the peak at the 8th week. (Fig5)

2.5. Fluorescence imaging techniques for retinal preparations

Compared with the normal group, the number of anti-IV+ collagen strands cross-linked between retinal vessels in the diabetes group began to increase on the 8th day after induction of DM. It was higher than that in the normal group at the 4th week with statistical difference (P < 0.05), and with the progress of
disease, there was a significant statistical difference (P < 0.01). The number of nerve cells in retinal tissue was decreased in diabetes group compared with that in normal group on the 8th day after induction (P < 0.05), but there was no statistical difference at the 4th week (P > 0.05). With the progress of the disease course, the number of nerve cells in retinal tissue decreased significantly in diabetes group at the 6th week (P < 0.01), and became worse at the 8th and 10th week, but the number of cells tended to be stable. In addition, the retinal tissue cell area of SD rats in the diabetes group was significantly higher than that in the normal group (P < 0.01) on the 8th day, but there was no statistical difference between the two groups with the progression of disease (P > 0.05). We observed that the retinal tissue vessels of SD rats in the diabetes group showed obvious tortuosity and local leakage at the 8th week, which was aggravated and the formation of neovascularization buds were observed at the 10th week. (Fig6)

2.6. The mRNA expression levels of VEGF, IL-6, CD18, ICAM and TNF-α in retinal tissues

The mRNA expression of VEGF in retinal tissue of diabetes group began to increase on the 8th day after induction, which was higher than that in the normal group (P < 0.05), and reached the peak at the 8th week. During the 10-week observation period, the mRNA expression of VEGF in the retinal tissue maintained a state of high expression in the diabetes group. At the same time, the mRNA expression of IL-6, ICAM and TNF-α in retinal tissue began to increase in diabetes group on the 8th day after induction, and the difference was statistically significant compared with that in normal group at the same time point (P < 0.05). With the progression of disease, the mRNA expression levels of IL-6, ICAM and TNF-α were in a state of high expression in the diabetes group, and a new peak appeared at the 10th week. However, CD18 mRNA expression in retinal tissue was significantly higher in diabetes group than that in normal group from the 4th week after model establishment (P < 0.01), and reached the peak at the 6th week after induction. (Fig7)

3. Discussion

It has become increasingly clear that diabetic retinopathy affects not only retinal vasculature, but also retinal neuronal and glial cells [11, 12] (Fig. 8). We found that the apoptosis of retinal neurocytes increased, microvascular injury and changes of microstructure in the early stage of DR. RGCs showed increased apoptosis during the fourth week of the diabetes course[6], and this early abnormality in neurons may result from the loss of neurocytes[13]. The earliest identified lesion in the diabetic retina is pericyte loss[7, 14]. Pericyte loss progresses over time to endothelial cell loss, resulting in the formation of acellular capillaries. As diabetes progresses, RGCs begin to decrease at 6th week[15]. However, previous report [16] suggested that ganglion cell became apoptosis after 12 weeks of STZ-induced diabetes. Based on the observation of different nodes between our study and previous reports [6, 16–19], oxidative stress and the high expression of inflammatory mediator precursors caused by hyperglycemia, the retinal ganglion cells apoptosis increased[20] on the 8th day after induction. Furthermore, there was a report suggested that the RGCs sizes were unchanged compared with the controls during the observation period of three months[16], which is consistent with our results except it in the 8th day after induction. We speculated that the oxidative stress and release of inflammatory mediator might cause the abnormal
metabolism of retinal neuronal cells in the early stage of diabetes, leading to the increase of retinal neuronal cells area on the 8th day after induction.

The morphological changes seen in small retinal vessels in DR include early loss of pericytes, loss of endothelial cells, increased vascular permeability, capillary dropout and vascular buds [21, 22]. These indicate the abnormal structure of retinal vessels and the occurrence of new blood vessels, which will lead to retinal neovascularizations. The earliest identified lesion in the diabetic retina is pericyte loss[7, 14]. Pericyte loss progresses over time to endothelial cell loss, resulting in the formation of acellular capillaries [23]. However, the mechanism of pericyte loss in early DR is unclear. Akagi et al [24] proposed that this mechanism is related to the sorbitol pathway, because they found that aldose reductase was present in human retinal capillary pericytes through immunohistochemical staining, but not in endothelial cells. A study[25] pointed out that platelet-derived growth factor B chain may selectively affect pericyte activity and lead to pericyte apoptosis. The loss of pericytes in the vascular wall can lead to the increase of E/P ratio, which will lead to the formation of microhemangioma.

In our study, we found that the VEGF-A concentration of vitreous and the expression level of VEGF RNA in the retinal tissue began to show high expression on the 8th day in the diabetes group, and reached the peak at the 8th week. Ayalasomayajula et al[8] found that the concentration of VEGF and VEGF mRNA expression in retinal tissue of diabetic rats were significantly higher on the 8th day after induction, which was consistent with our results. In the 4-week or 12-week observation period, VEGF concentration and VEGF RNA expression in retinal tissue of diabetic rats at the 4th week were the highest[9, 26]. However, VEGF-A concentration in vitreous cavity and VEGF RNA expression level in retinal tissue at the 8th week in our study were different from the study[26], which may be due to the sharp up-regulation of VEGF-A concentration and VEGF RNA expression level in diabetic rats caused by temporary acute high blood glucose concentration, ischemia and hypoxia in diabetic group. In addition, the overproduction of VEGF is associated with altered angiogenesis and increases in the permeability of retinal capillaries, resulting in retinal dysfunction[10]. According to these studies, the presence of new vessels and vascular buds may be inevitable, and the increasing vascular permeability appears to be a natural development of the disease process. One study reported that one of the earliest morphological changes exhibited in the retinal vasculature after DM induction was an increase in the number of lectin-negative and type IV + collagen strands containing no cellular elements and lacking any signs of endothelial proliferation as early as one and four weeks subsequent to STZ administration[9]. The type IV + strands suggest a possible association between vascular regression and the very early stages of DM, as reported in a study by Zhang et al[27]. These results suggest that endothelial cell degeneration and vascular basement membrane residue caused by diabetes are due to vascular degenerative changes.

Many reports had indicated that retinal microvascular injury has been linked to up-regulation of several cytokines such as IL-6, TNF-α, VEGF and CD18 and pathological over-expression of intercellular and vascular cell adhesion molecules (ICAM-1 and VCAM-1)[28–31]. Our study found that the mRNA expression levels of IL-6, ICAM and TNF were always high in the diabetes group during the 10-week observation period, and the high expression levels began to appear on the 8th day after the induction of
DM, but the mRNA expression level of CD18 began to increase significantly at the 4th week after induction, and reached the peak at the 6th week. Increasing evidence suggests that the IL-6 signaling pathway plays a prominent role in the endothelial cell dysfunction and vascular inflammation of DR [32–35]. A report [36] have indicated that the levels of tumor necrosis factor-α (TNF-α) and IL-6 in diabetes group were significantly higher than those of normal group in 2th week after induction, while another study [37] suggested that IL-6 and TNF-α became significantly elevated in diabetic retina after STZ injection as compared with normal rats in 4th week after induction and continued to 8th week. Our results are similar to those of the two reports. In addition, CD18 expression increased of retina from 1-week old diabetic rats [38]. Elevated levels of CD18 on neutrophils were present in each stage of DR, while the more severe the disease, the higher the level was [39]. The leukostasis is known to be increased in retinal blood vessels in diabetes, and this process is mediated via ICAM-1, while the retinal ICAM-1 levels were significantly increase when compared with nondiabetic controls after 1 week of diabetes [40]. ICAM-1 is upregulated by several stimuli, including VEGF, PARP activation, oxidative stress, and dyllipidemia [41–43]. Our study is a continuation of the above studies, and has similarities. At the same time, it is also to have a thorough and detailed understanding of the pathological changes in the early stage of DR.

In conclusion, we have provided evidence that STZ-induced diabetes amplify the levels of VEGF as well as IL-6, CD18, ICAM and TNF-α in the diabetic retina during the observation period of 10 weeks. We also observed not only the decrease of RGCs, microvascular injury and changes of microstructure began to occur in 8th day after induction, but the vascular permeability and neovascularization buds began to happen in 8th week. The results are sufficient to warrant further investigations, offer new insight into the pathogenesis of diabetic retinopathy, and offers novel targets to inhibit the ocular disease.

**Declarations**

**Funding information**

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**Conflicts of Interest**

The authors declare that they have no conflicts of interest.

**Availability of data and materials**
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

**Ethics approval**

Procedure of animals was in accordance with the Association for Research in Vision and Ophthalmology’s Resolution for the Use of Animals in Research, and the ARRIVE guidelines, which were approved by the First Affiliated Hospital of Nanchang University.

**Consent for publication**

Not applicable.

**Authors’ contributions**

Ang Xiao: Data curation; Formal analysis; Writing-review & editing;

HuiFeng Zhong: Data curation; Methodology; Writing-review & editing.

Lei Xiong: Formal analysis; Technical support; Writing review & editing.

Lin Yang: Methodology; Investigation; Writing-review & editing.

YunFang Xu: Formal analysis; Data curation; Writing-review & editing.

Yi Shao: Methodology; Writing-review & editing.

Qiong Zhou: Formal analysis; Funding acquisition; Investigation; Project administration; Supervision; Validation; Writing-review & editing. All authors read and approved the final manuscript.

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**Figures**
**Figure 1**

Example of the early stage of DR was examined on fundus camera (A) and fluorescence fundus angiography (B).

**Figure 2**

Morphological changes of retina in FFA. Figure A–E represent FFA examination images of diabetic rats and figure F–J represent it of normal rats at the 8th day, 4th week, 6th week, 8th week and 10th week after induction, respectively. Yellow arrow indicate vascular tortuosity and dilation, blue arrow indicate rough and leakage of vascular, and red arrow indicate typical leaky vessels with vascular tortuosity and dilation. Scale bar: 200µm.
Figure 3

The number and area of RGCs in diabetes group and normal group of retinal tissue HE staining (magnification, x400). Figure A–E are the HE staining of the diabetes group at the 8th day, 4th week, 6th week, 8th week and 10th week after induction, respectively, while figure F–J represent HE staining in normal group at corresponding time points. Figure K and L represent the ratio of RGCs and ganglion cell area of rats in the diabetes group and normal group at each time, respectively (*P < 0.01, #P > 0.05, **P>0.05). Yellow arrows indicate neovascularization buds and red arrows indicate abnormally dilated microvessels. Scale bar: 25µm.
Figure 4

The ratio of E/P ratio and number of acellular strands in diabetes group and normal group in PAS staining of retinal tissue (magnification, x400). Figure a–e are the PAS staining of the diabetes group at the 8th day, 4th week, 6th week, 8th week and 10th week after induction, respectively, while figure f–j represent PAS staining in normal group at corresponding time points. Figure K and L represent the ratio of E/P and acellular strands of rats in the diabetes group and normal group at each time, respectively (*P<0.01, #P<0.01). Yellow arrows indicate acellular strands and red arrows indicate neovascularization buds. Scale bar: 25µm.
Figure 5

The ratio of VEGF-A expression in vitreous cavity of SD rats in diabetes group and normal group (@$P<0.01$, $P<0.05$, @@$P<0.01$, $$P<0.05$).

Figure 6

Figure A–E are the immunohistochemical imaging of the diabetes group and figure F–J of it in normal group at the 8th day, 4th week, 6th week, 8th week and 10th week after induction, respectively (magnification, x100). Figure K, L and M represent the ratio of type IV collagen-positive strands, retinal cells and the area of retinal cells in the diabetes group and normal group at each time, respectively. Yellow arrows indicate type IV collagen-positive strands, blue arrows indicate vascular permeability, and red arrows indicate vascular buds. (*$P<0.05$, **$P<0.05$, #$P<0.01$, ##$P<0.01$, ###$P<0.01$, ***$P<0.01$). Scale bar: 75µm.
Figure 7

Figure A–E represent the ratio of VEGF RNA expression, the ratio of IL-6 RNA expression, the ratio of ICAM RNA expression, the ratio of TNF-α RNA expression and the ratio of CD18 RNA expression in retinal tissues, respectively. (*P < 0.05, **P < 0.01, ***P < 0.01, #P < 0.05 and ##P < 0.05).
Figure 8

Diagram of the development of DR. The pathogenetic process and molecular mechanism of the early occurrence and development of DR are shown in the dotted line frame.