VEGF, HIF−1α and PEDF expression in the retina of streptozotocin–induced diabetics rats treated with ozone

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Foundation item: Supported by Natural Scientific Research Fund of the Xinjiang Uygur Autonomous Region in China (No. 2014211C046)

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Received: 2014–11–25 Accepted: 2015–08–20

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Abstract

- AIM: To study vascular endothelial growth factor (VEGF), pigment epithelium derived factor (PEDF) and hypoxia inducible factor − 1α (HIF−1α) expression in retinal and serum in oxygen−treated streptozotocin (STZ)−induced diabetic rats and to determine the possible efficacy of ozone therapy for diabetic retinopathy (DR).

- METHODS: Seventy male Sprague−Dawley rats were used. Group A (n=10) received a normal diet, diabetic molding established by intraperitoneal injection of STZ (50mg/ml), then divided into three groups, group B without any intervention; and groups C and D given oxygen and ozone clyster treatment respectively, twice per week for 1mo. Retina and blood were taken under general anesthesia. Reverse transcription−polymerase chain reaction (RT−PCR) and enzyme−linked immunosorbent assay (ELISA) methods were used to study retinal and serum VEGF, HIF−1α and PEDF expression.

- RESULTS: VEGF occurred mostly in the inner layer of the retina; the difference of VEGF in the retina among each group has statistical difference (F = 23.923; P < 0.000), in which, group D closer to group A, but still has statistical difference (P < 0.05); there is no difference between group B and C (P > 0.05) except for no difference between group A and D (P > 0.05), others as same result as retinal VEGF expression. HIF−1α expression decreased in oxygen−treated rats (group D) compared with control group (P < 0.05); the difference between group A and groups B and C was significant (P < 0.05), and the difference between groups B and C with no statistical difference (P > 0.05). Overall, PEDF expression was lower than VEGF and HIF−1α, and groups A and D showed more expression than groups B and C, but the differences were not significant (P > 0.05).

- CONCLUSION: Ozone administration can reduce the
VGEF and HIF-1α expression and ozone may have potential uses in its treatment.

- **KEYWORDS:** diabetic retinopathy; ozone; vascular endothelial growth factor; pigment epithelium derived factor; hypoxia inducible factor-1α

**Citation:** Xie TY, Zhang CL, Chen XY. VEGF, HIF-1α and PEDF expression in the retina of streptozotocin-induced diabetic rats treated with ozone. Gaoyi Yanke Zazhi (Int Eye Sci) 2016; 16 (2):195–200

**INTRODUCTION**

Diabetic retinopathy (DR) is the leading cause of blindness in the western world and it affects approximately 75% of diabetic patients within 15y after the onset of diabetes[1]. DR is difficult to treat because the pathological changes prior to the clinical diagnosis has been made. The mechanism of DR is being investigated but it is complicated. Most animal and human trials have established that the pathological changes of DR result from hyperglycemia, which causes the concentration of glucose increase in cells and sorbitol (a mesostate) to aggregate. This disturbs the oxidation-reduction reaction balance and causes vessel occlusion. Ultimately, hypoxia and ischemia occur in the retina. Hypoxia and ischemia in the retina can activate transduction channels (such as protein kinase C or mitogen-activated protein kinase), and the endothelial function and pericyte cells are damaged[2]. Vascular endothelial growth factor (VEGF) plays a key role in the pathological changes of the retina in DR. VEGF genes are sensitive to hypoxia, which can lead to a strong response to hypoxia by up-regulating the hypoxic inducible factor-1α (HIF-1α) transcription factor. The HIF-1α subunit contains binding sites for the hypoxia response element, and through this site, it affects the target genes and its products such as cAMP - response element binding protein. Recent research has shown that HIF-1α is a major determinate of cellular VEGF expression and secretion. In addition to VEGF, the retina secretes other growth factors, such as pigment epithelium derived factor (PEDF), which contributes to the angiogenic homeostasis in ocular tissues. PEDF has been shown to be an endogenous antagonist of VEGF[3].

Hypoxia and ischemia are involved in the entire DR onset process. High concentrations of oxygen in the early stage of DR can reverse blindness, suggesting that hypoxia plays an important role in the process of DR[4,5]. Ozone may be an alternative treatment for DR, and several controlled trials have examined the feasibility and efficacy of using ozone as a therapeutic agent for the treatment of several diseases[6-7]. Ozone can increase the oxygen unloading capacity of hemoglobin in diabetic patients[8]. Thus, the main purpose of this research is to determine the role of ozone administration in improving oxidative stress in streptozotocin (STZ) - induced diabetic rats by examining VEGF, HIF-1α and PEDF expression, to establish its potential use in the strategy for the treatment of early-stage of DR.

**MATERIALS AND METHODS**

**Animals and Groups** Seventy male Sprague–Dawley rats weighting 300 – 320 g were purchased from Xinjiang Medical University Experimental Animal Center [ License No. SCXK (Xin) 2003–0001, China]. All experimental methods and animal care procedures were approved by the animal care committee of the Xinjiang Medical University (protocol IACUC - 20120523007), in accordance with the China Council on Animal Care. And then those rates were randomly divided into four groups as follow: group A; 10 rats just give the general feeding.

Others 60 rats were given high sugar and fat food for 1mo and then using streptozotocin (STZ, 10g/1, Sigma company, USA) intraperitoneal injection by concentration of 30 mg/kg, 3d and 1wk after, to testing the blood glucose by tail cutting respectively, blood glucose more than 16.7 mmol/L each time were be considered that the modeling established. And STZ-induced rats were randomly divided into three groups as follow in the process of making model there were 5 rats died for hemorrhagic shock. Group B (n = 20): STZ model, without intervene but given high fat and sugar diet. Group C (n = 20): STZ model + oxygen, given oxygen by concentration of 50 µg/kg twice per week. Group D (n = 20): STZ model + ozone, given ozone clyster as same concentration and methods as group C.

Under anesthesia by inject into abdominal cavity of chloral hydrate (made in Xinjiang Medicial University Pharmaceutical Preparation Room) by 0.3ml/100 g concentration. Enucleation of the eyeball and remove the anterior segment and part vitreous, put the retinal in liquid nitrogen (−80°) prepare for isolate RNA. And then take the blood from aortaventralis.

**RNA Isolation and Reverse Transcription – Polymerase Chain Reaction Analysis** Total retina RNA was extracted and purified by one step Trizol, according to the manufacturer’s instructions. The list of primers for each samples is as follows (Table 1), cDNA were generated from 1 µm of total RNA, using the High-Capacity cDNA Reverse Transcription Kit (Thermo Corp., USA), and subjected to a 40 cycle polymerase chain reaction (PCR) amplification. Three replicates were run for each sample in a 96–well plate electrophoresis to identification the PCR amplifications products.

**Enzyme–Linked Immunosorbent Assay** VEGF levels in the serum of rats were estimated with enzyme–linked immunosorbent assay (ELISA) kit (VEGF Rat ELISA Kit, abcam, Lot: GR148932 − 1, batch number: ab100786) according to the introduction to perform the test.

**Statistical Analysis** The results were analyzed using SPSS 17.0. The data are presented as the x ± s when a normal distribution was found. Parametric data were analyzed using ANOVA, and VEGF, HIF-1α and PEDF mRNA expression
levels in two groups were compared using the LSD t-test. Significance was defined as \( P<0.05 \).

**RESULTS**

**General Conditions**  Weight were decreased and blood glucose were increased steady in the whole rats after modeling estimated, with diabetic duration extended, the blood glucose in each group keep high levels in the same time point. In another word, ozone and oxygen has no influence on blood glucose. (Figure 1A, B)

**Immunohistochemical Determination of VEGF**  Retinal VEGF expression was identified by brown staining. VEGF expression in the control group was negative or weakly positive with a small amount of perivascular expression. VEGF expression was higher in the other groups, especially in the sub-limiting membrane, retinal ganglion cell (RGC) layer and the inner and outer plexiform layer. Group D showed a similar expression patterns to, but lower expression levels than group B and group C (Figure 2).

**VEGF Expression in Each Group**  Reverse transcription–polymerase chain reaction (RT–PCR) results show that VEGF was detectable in all retina samples. The expression of VEGF mRNA in group A, B, C and D is 4.22±1.18, 11.60±3.42, 10.75±2.81 and 7.40±2.13 respectively, and there is statistic significance (\( F=23.923; P=0.000 \)). VEGF expression in group B were nearly three times more than group A (\( P=0.000 \)); expression of VEGF in group D were less than group B and C, but more than group A (\( P=0.000 \)); there is no statistical significance between group B and C (Figure 3).

ELISA methods get the same results as RT–PCR and the VEGF level in group A, B, C and D, they are 4.29±1.11, 12.1±1.62, 10.65±1.58 and 4.44±1.76 respectively, there is statistical significance (\( F=62.249; P=0.000 \)) and VEGF level in group B and C were more than group A and D (\( P=0.000 \)); and there is no statistical significance between group A and D (\( P=0.997 \)) as well as group B and C (\( P=0.873 \)) (Figure 4).

**HIF–1α mRNA Expression**  HIF–1α mRNA expression by RT–PCR in group A was 2.3±0.3, which was significantly different from expression levels in groups B and C (\( P<0.05 \)). Similar expression levels were found in group B and C (2.8±0.3 and 2.8±0.5, respectively; \( P>0.05 \)). The lowest expression was found in group D (1.8±0.4), which was significantly different compared with group A (\( P<0.05 \)) (Figure 5).

**PEDF mRNA Expression**  PEDF mRNA expression in each group was follow; 0.22±0.12 in group A, 0.13±0.08 and 0.12±0.07 in groups B and C, and 0.19±0.09 in group D. Although PEDF mRNA expression was different in each group, the differences with no statistically significant (\( F=2.803; P=0.05 \)) (Figure 6).

**DISCUSSION**

DR is considered to be an ischemic and hypoxic disorder that can lead to neovascularization and blindness. In the hypoxic state, it is thought that the retina produces growth factors that lead to vessel leakage and macular edema, angiogenesis causes fibrovascular tissue formation which can cause retinal detachment and finally loss of vision.

Retinal pathological changes that occur in STZ–induced rats vary with different tests. Some researchers found evidence of hypoxia in the diabetic mouse at a much earlier time point of 5mo using the oxygen – dependent probepenimidazole\(^9\). Others have found vasoconstriction and a substantial decrease in blood flow is as early as after 3–4wk of diabetes in both mice and rats. Thus, hypoxia plays a vital role in the process of DR, but the exact point at which hypoxia occurs in the histology is still undefined. Research has shown that capillary non–perfusion is representative of hypoxia in the tissue\(^{10}\), and this may explain why the most clinical symptoms are posterior to the histopathology changes. Our study showed that VEGF occurred in each layer of the retina in 1mo STZ–induced rat by immunohistochemical determination. There is no clinical symptom but the pathology changes occurring. It means that we should pay attention to the diabetic patients whose without obvious eye symptoms.

VEGF plays a key role in the DR process has been established\(^{11-13}\). VEGF is a protein that is a vascular endothelial cell mitogen and vascular permeability factor, and
Figure 2  VEGF expression by immunohistochemistry, VEGF is indicated by the brown stain  A: Group A with VEGF immunohistochemical staining, VEGF expression is hard to detected (DAB×400); B: Group B with VEGF expression has shown in GCL of retina (DAB×400); C: Group C with VEGF stained in the GCL and OPL (DAB×400); D: Group D with a low level of VEGF expressed in the GCL (DAB×400); GCL: Ganglion cell layer; OPL: Outer plexiform layer.

Figure 3  VEGF expression in each group in retinal GB: Group blank; GM: STZ–induced model group; GO2: STZ–induced rats with treatment by O2; GO3: STZ–induced rats with treatment by ozone.

Figure 4  HIF–1α expression in each group  GB: Group blank; GM: STZ–induced model group; GO2: STZ–induced rats with treatment by O2; GO3: STZ–induced rats with treatment by ozone.

Figure 5  PEDF expression in each group  GB: Group blank; GM: STZ–induced model group; GO2: STZ–induced rats with treatment by O2; GO3: STZ–induced rats with treatment by ozone.

It is the most direct neovascularization factor, because VEGF mRNA expression increased during retinal hypoxia and remained elevated during the development of neovascularization. Animal studies revealed that VEGF synthesis occurs within the retina[14], which is consistent with the results of our study. Immuno–histochemical staining showed that VEGF mostly occurred in the inner layer of the retina. The VEGF content in the retina was significantly increased in groups B and C, which was different from the control STZ–induced rats. VEGF expression in ozone–treated rats (group D) was significantly lower than in the STZ–control group (group B) and in the oxygen treated group (group C). Meanwhile, the research show that even in the serum get the same results, that is VEGF can be detected in each group. But the model group and oxygen group was much
more express than control group, just as other researches that VEGF no matter in retinal or in serum are involve in the process of the DR. To the contrary, the VEGF in retinal or in serum level decreased obviously compared to the model group or oxygen group, it means that ozone can reduce VEGF expression, whether it through antibiotic or improve oxygen supply still unknown, but it really worked.

VEGF is regulated by HIF–1α. Diabetics is a disorder that is associated with oxidative stress; research shown that HIF–1α is the primary hypoxia signaling protein in cells for regulating angiogenesis, and that it induces the transcription of several genes. Oxygen plays a key role in stabilizing HIF–1α and its function. When oxygen is normal, HIF–1α isodioxidatively modified by prolyl 1 hydroxylase enzymes, but when cells are in hypoxia, the proline is not hydroxylated and HIF–1α escapes degradation. Thus, HIF–1α is a major determinant of VEGF cellular expression and secretion. Our study shows that HIF–1α expression in the model groups (groups B and C) was higher than the control group (group A). This is in agreement with the hypoxia results and is an initial change. However, after 1mo of ozone treatment, HIF–1α expression in the STZ+ozone group (group D) was even lower than that in the control group (group A). This may be because ozone consists of three oxygen atoms; the free oxygen can combine with another oxygen atom, and in this way improve the oxygen supply, which exceeds that in the oxygen treatment group. For the single methods to test HIF–1α, so it’s real but the exact mechanism require further study.

PEDF is another growth factor that is secreted by the retina, and it contributes to the angiogenic homeostasis in ocular tissues. PEDF was initially identified as a neurotrophic factor that is secreted by human fetal retinal pigment epithelium (RPE) cells. However, in the eye, studies showed that endothelial quiescence and barrier function are achieved through a balance of VEGF and PEDF. During the onset of active DR, VEGF and PEDF expression were reversed. Our study showed that low PEDF levels were expressed compared to that of VEGF and HIF–1α. Though there is no difference among each group of PEDF expression, however, the PEDF level in ozone group close to the blank group, and the group B and C still keep lower level of PEDF. As an angiogenic inhibitor, the function of PEDF is more universal because it can activate more tissues than other inhibitors, but the effects of angiogenic inhibition were dose-dependent, so the percentage of PEDF should be increased to the extent. Low levels of PEDF performance in our study, which maybe ozone did not work by this way or the period too short for PEDF to have an effect on the retina.

Ozone, in theory, has oxidizing action that leads to the formation of hydrogen peroxide, which enters cells with various effects; in red blood cells, it shifts the hemoglobin dissociation curve to the right and facilitates release of oxygen; and in leucocytes and endothelial cells, it induces the production of interleukins, interferon, transforming growth factor (TGF), nitrogen oxide and antacoids. Previous studies have demonstrated that controlled ozone administration may promote an oxidative preconditioning or adaptation to oxidative stress, thus preventing the damage induced by reactive oxygen species (ROS). It has also been shown that ozone therapy can effectively reduce hypoxic and ischemic diseases, such as diabetic complications (for example, diabetic foot and diabetic nephropathy, and ischemia–reperfusion disease). There is been no previous research on using ozone to treat DR, but ozone may be beneficial for the DR hypoxia and ischemia.

In our study, we found that ozone can reduce the VEGF and HIF–1α expression, and increase PEDF expression to maintain the RPE barrier function. Although oxygen is effective for treating hypoxia, we found that a general oxygen concentration did not seem to work to treat DR. It is possible that effectively treating hypoxia diseases required a high percentage of oxygen.

Many physicians are worry about the side–effects of ozone and they refuse to use it. In fact, the side–effects can be avoided if an ozone generator is used correctly. The duration of our study was short, and a longer treatment and research duration may yield more information. However, our research still suggests that ozone can be used effectively to treat patients with DR. Further research is required to translate these results from the bench to the clinic.

REFERENCES


