·Basic Research·

Histopathological changes in retinas and F-ERG features of streptozotocin-induced diabetic rats treated with ozone

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Abstract

• **AIM: To study the histopathological changes in the retina and flash electroretinogram (F-ERG) features of ozone-treated streptozotocin (STZ)-induced diabetic rats.**

• **METHODS: Seventy male Sprague Dawley rats were grouped as follows: blank group (GB, *n*=10), model control group (GM, *n*=18), ozone group (GO_3, *n*=19), and oxygen group (GO_2, *n*=18). The model was induced by single intraperitoneal injection of STZ. Ozone or oxygen enteroclysm was given twice per week for 4wk. F-ERG and histopathological examinations were performed one month after treatment.**

• **RESULTS: Under dark adaption, as compared to GB, the other groups each had differential decreases in the a-wave amplitudes (*P*<0.05); the latencies were delayed in GM, GO_2, and GO_3 rats (*P*<0.05). Similar results were observed under light adaption, with the exception that the a-wave of the amplitudes (*F*=0.28, *P*>0.05). There were significant differences in the apoptosis index among the groups (*P*<0.05). Under ozone treatment, apoptosis was decreased in GO_3 as compared to GM and GO_2.**

• **CONCLUSION: Ozone administration alleviates nerve damage and reduces pathology and apoptosis in the retinas of diabetic rats.**

• **KEYWORDS:** diabetic rat retina; ozone treatment; histopathological changes; flash electroretinogram features

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INTRODUCTION

Diabetic retinopathy (DR) is a major cause of blindness among the working-age population in developed countries [1]. Research has estimated that the prevalence of DR would reach to 19.99 million globally by 2030 [2]. DR is therefore a significant health problem. Currently, treatments for DR include laser therapy, anti-vascular endothelial growth factor (VEGF), and vitrectomy. However, these treatments are all adapted to the middle or later phases of DR. Treatments for early stage of DR remain controversial.

Ozone is a molecule consisting of three atoms oxygen. it is dynamically unstable structurally due to the presence of mesomeric states [3]. It can be used as a strong oxidant and a free radical scavenging antioxidant activation system, and has been shown to affect oxidized glutathione reductase activity and to enhance metabolism[4]. Currently, a variety of diseases including ischemic disease [5], autoimmune diseases, and age-related macular disease (AMD-dry) [6] are treated effectively with ozone.

Given that diabetes is known to promote oxidative damage and that ozone can protect cells in oxidative stress situations, we studied the action of ozone in streptozotocin (STZ)-induced diabetic rats by examining histopathological changes and flash electroretinogram (F-ERG) features. We hope to establish the use of ozone as a potential theraputic strategy for treatment in the early stages of DR.

MATERIALS AND METHODS

Animals 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. 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]. Adaptive feeding was carried out for 4wk under controlled temperature (23℃), humidity (50%), and lighting (12-hour light/dark cycle).

Diabetic Model Total 60 rats were selected randomly using a table of random numbers to receive a high fat and sugar diet for 45d. Blood sugar and weight were monitored each week. The model of diabetic rats was induced by one instance of fast abdominal injection of STZ (30 mg/kg, dilution with citrate buffer 0.1 mmol/L, pH4.3-4.5, Sigma).

Blood samples were taken from the tail to test blood glucose levels at 24h and 7d respectively after injection (Surestep glucose meter, Johnson & Johnson, USA). Diabetes mellitus (DM) rats were defined as those with random blood glucose levels greater than 16.7 mmol/L at both the 24h and 7d measurements. Five rats died of hemorrhagic shock following the STZ injection. In total, 55 diabetic rats model were successfully generated.

Groups Model control group (GM, n=18): fed continuously with a high fat and sugar diet. Ozone group (GO_3, n=19): received enteroclysm with ozone [5] (ozone generator, HealOzone, Company Kawo, Germany) at 50 μg/kg, twice per week, for one month. In brief, after evacuating the rectum of the rats using 1 mL syringe to a depth of about 4 cm, we injected 50 mg/L ozone (mixed gas with ozone and oxygen) and then pressed the anus for 5min to prevent gas leakage. Oxygen group (GO_2, n =18): rats received an equivalent dose of oxygen, administered as GO_3 group. Blank group (GB, n =10) without any treatment.

Flash Electroretinogram After anesthesia, rats were warmed with homemade cloth wraps. After 60min of dark adaptation, the pupils were completely dilated with tropicamide eyedrops. Reference electrodes were placed subcutaneously at ipsilateral cheek. Grounding electrodes were placed subcutaneously at tail with a hypodermic needle. Ophthalmic gel was used on the surface of the eyeball to protect the cornea, gold ring electrodes were applied on the cornea for measurement. Electrode impedance was controlled within 10 Ω (placing the electrodes under weak red light). All operations were performed by a single individual to prevent error. F-ERG were administered with a white flash of 3.0 cd·s/m^2, for an interval of 15s, passband 0.1-500 Hz with 250ms scanning time, 4 times superposition. Oscillary potentials (Ops) were administered with a white flash of 2.398 cd·s/m^2, a flash interval of 15s; passband 100-500 Hz with 250ms scanning time, 8 times superposition. The latency and amplitude of a-wave and b-wave were tested.

TUNEL After aneshesia, eyeballs were dehydrated, permeabilized with xylene, embedded with paraffin, and prepared as consecutive slices. Microwave repair was carried out for 10min before adding 0.01 mL citrate solution and cooling. TUNEL mixture (1:30 dilution, TUNEL Apoptosis Kit, Roche, Switzerland) was added at 37℃ in a wet box and incubated for 60min. After adding chromogenic DAB, the samples were then washed, dehydrated, permeabilized, and sealed. Optical microscopy (Leica,LeicaMicrosystems Wetzlar GmbH, Germany) was used to determine the apoptosis index (AI), which was defined as the percentage of positive cells out of the total number of mononuclear cells.

Table 1 Amplitudes and latencies of a-waves and b-waves in the dark adaption $\bar{x} \pm s$

Groups	Amplitudes (μV)		Latencies (ms)	
	a-wave	b-wave	a-wave	b-wave
GB	39.61±1.30	99.45±2.77	13.44±2.55	41.78±1.99
GO_3	18.68±0.92	46.86±2.53	19.81±2.71	42.91±4.72
GO_2	10.44±0.97	32.91±2.61	30.10±2.42	53.40±4.97
GM	10.72±1.06	33.43±2.76	30.30±2.58	54.60±2.27
F	1543.43	1548.69	98.31	31.17
P	<0.05	<0.05	<0.05	<0.05

GB: Blank group; GO_3: Ozone group; GO_2: Oxygen group; GM: Model control group.

Table 2 Amplitudes and latencies of a-waves and b-waves in the bright adaption $\bar{x} \pm s$

Groups	Amplitudes (μV)		Latencies (ms)	
	a-wave	b-wave	a-wave	b-wave
GB	16.17±1.37	25.41±1.25	12.77±1.71	43.00±2.82
GO_3	15.79±1.45	21.82±0.78	15.72±1.90	51.63±2.87
GO_2	16.28±1.31	15.01±0.87	15.40±2.01	65.20±1.54
GM	15.86±1.44	14.18±0.79	15.30±1.63	65.30±1.94
F	0.28	327.02	5.20	203.62
P	>0.05	<0.05	<0.05	<0.05

GB: Blank group; GO_3: Ozone group; GO_2: Oxygen group; GM: Model control group.

Statistical Analysis The data were analyzed using SPSS17.0 and presented as mean±SD. One-way ANOVA was used to compare the differences between groups and LST-D test used for pairwise comparison. P <0.05 was considered statistical significance.

RESULTS

Flash Electroretinogram As compared to the amplitudes of GB (39.61±1.30 μV for a-wave and 99.45±2.77 μV for b-wave), the other three groups had decreased values. The variation between GM and GO_2 was statistically significant (P<0.05). Similar changes were observed for the latency among the groups. As compared with the GB group, the latency in the other treatment groups were delayed. The latency of the GO_3 group was slightly delayed, while the latency in the GM and GO_2 groups were more severely delayed; the difference in latency between GO_3 and GO_2 or GM groups was statistically significant (P<0.05) (Table 1).

In the bright adaptation experiments, the amplitude of a-wave did not differ significantly between groups (P>0.05). However, there were statistically significant differences between the groups in the amplitude latencies of b-wave, and in the latencies in a-wave (P<0.05) (Table 2).

Apoptosis in the Retina Apoptosis was present in each group, but was obvious in GM, GO_2, and GO_3 groups. Especially in retinal ganglion cells, inner nuclear layer (INL), and the vascular endothelial cells, apoptosis was obvious but seldom in the outer nuclear layer (Figure 1). The apoptosis index for each group was as 1.97±0.53 in GB,

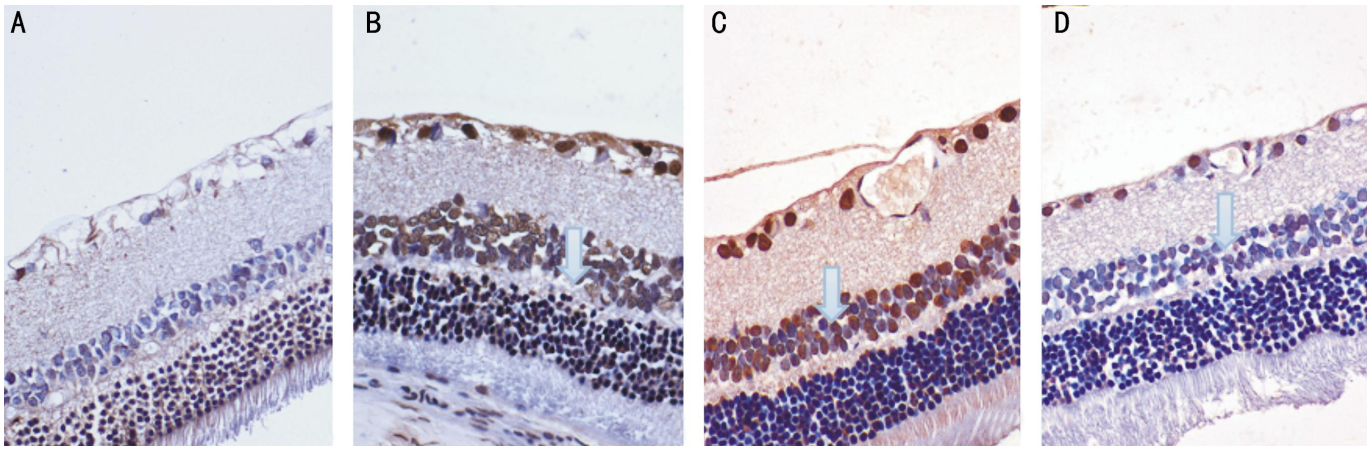

Figure 1 Apoptosis of the retina cells (arrows) in each group by TUNEL A: Infrequent occurrence of TUNEL positive cells in GB; B: TUNEL positive cells seen in GCL and INL in the GM group; C: In GO_2, TUNEL positive cells was as the same as GM ; D: In GO_3 group, a few TUNEL positive cells were seen in GCL and INL, more than in GB but less than in GO_2 and GM (×400).

34.43±5.56 in GM, 19.22±3.30 in GO_3, and 34.68±5.80 in GO_2 (F=89.07; P<0.05). ANVOA test show that there has statistically significant among each group(F=4.65; P<0.05); pairwise comparison indicated that except for the difference between GO_2 and GM (P>0.05), difference between other groups was statistically significant (P<0.05).

DISCUSSION

Extensive research efforts have confirmed that the nerve degeneration of the retina in DR occurs prior to clinical manifestations of the disorder. Normal vision depends on the integrity of the retinal neuron network signal transduction pathways, which depend on interactions among undamaged neurons, glial cells, blood vessels, and epithelial cells. Due to the interdependence among these cells, any degeneration of a particular subgroup can damage the whole functionality of the retina. Nerve damage associated with DR leads to irreversible blindness. Therefore, it is necessary to develop methods to intervene in the initial impairment of nerves in the retinas of the DR patients.

The oxidizing action of ozone leads to the formation of hydrogen peroxide which then enters into cellsand leads to various effects. In red blood cells, ozone shifts hemoglobin dissociation curves to the right and facilitates the release of oxygen[7-8]. In leucocytes and endothelial cells, ozone induces the production of interleukins, interferons, transforming growth factor, nitrogen oxide, and antacoids [9-10]. Previous studies have confirmed that controlled ozone administration may promote an oxidative preconditioning or an adaptation to oxidative stress, prevent the damage induced by ROS[11]. Therefore, we wanted to verify whether ozone therapy can be used as a method to prevent nerve damage of the retina during the early stages of DR.

Electroretinogram (ERG) can identify electric response of nerves induced by visual stimulation. It has been found that a decline in retinal function may occur prior to the observable vascular lesions in DR by using of ERG [12-14]. The results indicated that the function of the inner retina is influenced primarily by diabetes mellitus as well. This manifested as the reduced amplitude and frequency of visual stimulations in the amacrine cells, and dutter oscillation was observed occurring in ERG results [15]. ERG can reflect the severity of nerve damage in DR. A-wave of ERG originate from the photoreceptor cell layer in retinal. These can be understood as a kind of hyperpolarization action potential that reflects the bioelectric activities of photoreceptor cells. ERG b-wave originate from bipolar cells of the retina, the cells that change the electrical activity of inner nuclear layer in retinal. ERG b-waves are considered to offer higher sensitivity and reliability index in the diagnosis of retinal function [16-17]. According to our results in the dark adaption experiments, the amplitudes of the a- and b-waves of the GM groups declined, and the latencies of a- and b-waves were delayed by more than two times compared to the GB group. In the bright adaptation experiments, with the exception of the a-wave of amplitudes for which there were no statistical differences among the groups, compare to GB, b-wave amplitudes decreased and a- and b-waves latencies delayed in other groups. These findings are in accordance with other studies that show damage mainly in the nerve layer in the early stage of DR. In our results, the F-ERG in the diabetic treatment groups changed compare to the control group GB, but there were differences among the treatment groups in both the dark and bright adaption experiments, the results for GO_3 were always close to those of GB. In other words, the nerve impairment in GO_3 was less severe than that in GO_2 and GM. This means that ozone likely decreases damage to the retina in the early phase of DR.

Histopathological evidence shows that retinal microangiopathy occurs in diabetic subjects well before the onset of retinal dysfunction and the appearance of clinically detectable retinopathy [18]. Research by Barber *et al*[19] confirmed that morphological changes occurred in the retinal

ganglion cells and microglial cells in 1-month diabetic model rats. Similar results were observed in our study, the pathological changes occurred after 2mo of the diabetic model in rats, as manifested by edema ganglion cells, vacuolation of rod cells, congestion in micrangium of the retina, and neovascularization. It is noteworthy that, as compared to the GO_2 and GM groups, the pathological changes in the GO_3 group were not obvious. Using light and electron microscopy, we found that the pathological changes were more severe in the GO_2 and GM groups than in the GO_3 group. Therefore, our study demonstrates that ozone, at least in part, can prevent structural alterations in the retina associated with DR.

Retinal cell apoptosis is an early indicator of DR[20]. Research has demonstrated that retinal capillary endothelial cells and pericyte cells are apoptosis in diabetic patients or experimental galactosemia rats (TUNEL positive)[21]. A large number of studies have shown that diabetes can induce retinal cell apoptosis, reduce the number of retinal ganglion cells, and cause inner plexiform layer atrophy. Diabetes can also reduce the number of remaining retinal neurons. Research has shown that with 7.5-month STZ induced diabetic rats, for example, the number of ganglion cells in the retina can be reduced by as much as 10% [19]. These studies have demonstrated that apoptosis is an important part of the pathogenesis in DR. The same results were observed in our study, apoptosis occurred in retinas of 2-month DM rats. Apoptosis was particularly obvious in the ganglion cells and INL. Our findings were similar to study of Bresnick[22], who revealed that a neuropathic pathogenesis mechanism may exist in DR. The apoptosis index varied between groups, and was significantly higher in the GO_2 and GM groups than in the GO_3 group. In other words, ozone treatment may prevent apoptosis of retinal cells to some extent.

Enteroclysis were adopt in this trial, compare to other methods of ozone treatment, such as ozone venous blood therapy, ozone balneotherapy, ozone radiation therapy, the dosage of enteroclysis is relatively hard to control. This method will effect the ozone function, but it is an method easy to perform, relatively safety.

In conclusion, using ozone in humans and animals is controversial because of its side effects. These are specifically related to the formation of free radicals and irritation of the respiratory system. However, as it has been established for clinical use in the treatment of other diseases, ozone may be an effective and economical treatment that can benefit many potential DR patients. Our study clearly suggests that ozone therapy is at least partially effective in maintaining retinal structure and function in diabetic rats. Other words, ozone appears to be a useful treatment during the early stages of DR and may be an effective treatment for maintaining visual acuity in DR patients.

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