Ozone Therapy Effects in the Oxidative Stress Associated to Diabetes Mellitus

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Abstract

It is well recognized the presence of oxidative stress in diabetes mellitus. Ozone can exert its protective effects by means of an oxidative preconditioning, stimulating and/or preserving the endogenous antioxidant systems. The aim of this paper is to evaluate the ozone effects, in a preclinical and preliminary clinical studies, in the oxidative stress associated to diabetes. Rats were divided in: 1- negative control group; 2- positive, using streptozotocin (STZ) as a diabetes inductor; 3- ozone, 10 treatments (1 mg kg-1), after STZ-induced diabetes and 4- oxygen (26 mg kg-1), as group 3 but using oxygen. Patients with diabetic foot were divided in 2 groups: ozone (using rectal and local ozone) and antibiotic (systemic and locally). Ozone treatment improved glycemic control and prevented oxidative stress associated to diabetes mellitus and its complications, in both studies, in agreement with the excellent results obtained clinically.

Introduction

Diabetes mellitus is characterized by metabolic abnormalities, a disorder of carbohydrate metabolism, with the presence of hyperglycemia and glycosuria, resulting from inadequate production or utilization of insulin. Long-term complications, that cause morbidity and premature mortality, is characterized by microvascular disease with capillary basement membrane thickening, macrovascular disease with accelerated atherosclerosis, neuropathy involving both the somatic and autonomic nervous systems, neuromuscular dysfunction with muscle wasting, embryopathy and decrease resistance to infections. Such chronic complications involve the eyes, kidneys, heart, nerves and blood vessels. Accelerated atherosclerosis produces 80 % of all diabetic mortality, three fourths off it owing to coronary disease. A more frequent concomitant of distal anesthesia is the development of neurotropic ulceration, particularly on the plantar aspect of the foot. Anesthesia leads to a worsening of

any minor injury because of the absence of protective painful stimuli. This problem in addition to pre-existing microvascular and macrovascular circulatory impairments characterizes the underlying mechanisms that may lead to rapid gangrene after foot injury (1,2).

It has been demonstrated, in diabetic patients, the role of the reactive oxygen species (ROS) with an increase oxidative damage at the level of lipid peroxidation, DNA injury and protein damage (3-5). Activation of polyol pathway, non-enzymatic glycosylation of proteins and the increase of ROS play an important role in diabetes complications (6,7). Also, a decrease in the antioxidant defense system, involving the erythrocyte superoxide dismutase and catalase (8,9), with a simultaneous decrease in vitamin C concentration in leukocytes (10) and a decrease in the scavenger capacity of radicals in plasma have been mentioned (11).

Ozone can exert its protective effects by means of an oxidative preconditioning, stimulating and/or preserving the endogenous antioxidant systems and by blocking the xanthine/xanthine oxidase pathway for ROS generation, as it has been demonstrated in the damage induced by carbon tetrachloride (CCl4) and in the hepatic and renal ischemia-reperfusion (12-15). Also, ozone oxidative preconditioning has been proven to preserve glycogen content and to reduce lactate and uric acid formation, controlling oxidative stress induced by CCl4 administration to rats (16). In addition, it has been demonstrated that endovenous ozone therapy, in patients with myocardial infarction, has a beneficial effect on blood lipid metabolism, decreasing blood cholesterol and provoking the activation of antioxidant protection system (17). Ozone has been used with good results in the treatment of patients with diabetic foot, taking into account its germicide properties and its influence in the processes of oxygen metabolization, besides other effects (18).

The socioeconomic impact of diabetes is devastating to individual patients and society as a whole. Any treatment that is capable to normalize oxygen metabolism, to modulate the oxidative stress and to have germicide properties can improve the quality of life of these patients, as well as diminish patient consumption of medicines. Taking into account the ozone therapeutical properties, the aim of this study is to evaluate the ozone effects in the oxidative stress associated to diabetes mellitus, using a preclinical and a preliminary clinical studies.

Materials and Methods

Preclinical study

Animals

Male Sprague-Dawley rats weighing 250-278 g, obtained from CENPALAB (Bejucal, Havana, Cuba), were used in this study (n = 40). Rats were housed in plexiglass cages, maintained in an air-filtered and temperature-conditioned (20 - 22 ^{o}C) room with a relative

humidity of 50 - 52 % and under an artificial light/dark cycle of 12 h. Animals were fed with standard laboratory chow and water *ad libitum*. All procedures were performed as approved by the International Animal Care Committees and in accordance with the European Union Guidelines for animal experimentation.

Induction of experimental hyperglycemia

Experimental diabetes was induced by a single intraperitoneal (i.p.) injection of 45 mg kg-1 streptozotocin (STZ) (Sigma, St. Louis, MO, USA) to overnight fasted rats (19). STZ was dissolved in citrate buffer solution (0.1 M, pH 4.5) and freshly prepared immediately before injection. Animals were considered hyperglycemic when non-fasting serum glucose levels were higher than 20 mM after 48 hours of STZ injection (20). Blood glucose was measured using a diagnostic kit obtained from Sigma 315-100 (Sigma, St. Louis, MO, USA) based on a colorimetric reaction.

Treatment

Ozone (O3) was generated by an OZOMED equipment manufactured by the Ozone Research Center (Cuba) and was administered by rectal insufflation. The O3 obtained from medical grade oxygen was used immediately and it represented only about 3 % of the gas (O2 + O3) mixture. The ozone concentration was measured using an UV spectrophotometer at 254 nm. The O3 dose is the product of the O3 concentration (expressed as mg l^{-1} and the gas (O2 + O3) volume (l). By knowing the body weight of the rat, the O3 dose is calculated as mg/kg (12-16). Rats received 4.5 - 5.0 ml of O3 (concentration 50 mg/l) by rectal insufflation, after 48 h of the induction of experimental diabetes with STZ.

Animals were allocated randomly to the following treatment groups (10 animals each): 1, control group, treated only with physiological saline solution; 2, positive control group, using STZ as a diabetes inductor; 3, ozone group, receiving 10 treatments (1.1 mg kg-1) one per day after 48 h STZ-induced diabetes; 4, oxygen (26 mg kg-1) one per day, as in group 3 but using oxygen only.

Sample preparation

24 h after the last O3 and O2 treatments, blood glucose was measured, body weight of the animals was monitored and then the animals were euthanized by ether anesthesia. Afterwards pancreas was promptly removed for biochemical studies (catalase, superoxide dismutase, glutathione, glutathione peroxidase, total peroxides and malondialdehyde). Pancreas homogenates were obtained using a tissue homogenizer Edmund Bühler at 4$_o$C. The homogenates were prepared using a 50 mM KCl/Histidine buffer pH 7.4, 1:10 (w/v) and were spun down with a Sigma Centrifuge 2K15, at 4^{o}C and 8500 x g during 20 min. Supernatants were taken for biochemical determinations.

Clinical study

Patients and Treatment

Adult patients of both sex, with diagnosis of diabetes mellitus, suffering of ulcers of the feet and lower extremities, were hospitalized in the Institute of Angiology and Vascular Surgery. This study was accepted by the Scientific and Etics Committees of the Institution. 20 patients (with informed consent) were divided, at random, in two groups of treatment: 1-, control group, 10 patients treated with antibiotic therapy (according to the germ present) systemic and locally in the lesion, with the conventional method of treatment and 2-, ozone group, 10 patients treated daily with ozone (generated by an OZOMED equipment), 20 sessions, by rectal insufflation (with an ozone dose of 10 mg, ozone concentration: 50 mg/l) and locally. For the local ozone treatment, the lesion was introduced in a plastic bag, sealed to the leg and then submitted to vacuum, in order to eliminate the air inside it. Afterward, the bag was refilled with ozone at a concentration of 80 mg/l. The patient remained with the plastic bag for 1 hour. After that, the bag was retired and the lesion was cured with ozonized sunflower oil (Oleozon^{R}).

To overnight fasted patients included in both groups, blood sample was extracted in the morning for biochemical studies, at the begining and 24 h after the last ozone and antibiotic treatments. Glucose, catalase, superoxide dismutase and lipid peroxidation were measured in this protocol.

Biochemical determinations

All biochemical parameters were determined by spectrophotometric methods using an Ultrospect Plus Spectrophotometer from Pharmacia LKB. Catalase activity was measured by following the decomposition of hydrogen peroxide at 240 nm at 10 sec intervals for one minute (21). Superoxide dismutase (SOD) and glutathione peroxidase (GPx) were measured using kits supplied by Randox Laboratories Ltd., Ireland (Cat. No. SD125 and No. RS505). Concentrations of malondialdehyde (MDA) were analyzed using the LPO-586 kit obtained from Calbiochem (La Jolla, CA). In the assay, the production of a stable chromophore after 40 min of incubation at 45^{o}C was measured at 586 nm. For standards, freshly prepared solutions of malondialdehyde bis [dimethyl acetal] (Sigma) were employed and assayed under identical conditions (22). Quantification of total hydroperoxides was measured by Bioxytech H2O2-560 kit (Oxis International Inc., Portland, OR, USA) using xylenol orange to form a stable colored complex, which can be measured at 560 nm. Total protein concentration was determined by the method of Bradford with bovine serum albumin as standard (23).

Statistical analysis

The OUTLIERS preliminary test for detection of error values was initially applied. Afterward, data were analyzed by one-way analysis of variance (ANOVA) followed by

homogeneity variance test (Bartlett-Box). In addition, a multiple comparison test was used (Duncan test). Results are presented as means ± standard deviation. The level of significance was accepted at $p<0.05$.

Results

Preclinical study

Rats treated with streptozotocin (STZ) and STZ + O2 were hyperglycemic and lost weight over the experimental period (Table I). Ozone treatment reduced hyperglycemia by 40 % in comparison with STZ-treated rats. Body weight of the rats was increased in a similar way as non-diabetic control.

Table I. Body weight and plasma glucose concentrations

Groups n = 10	Body weight changes (g)(1)	Plasma glucose (mmol/L)		Significance of plasma glucose
		Start	End(2)	
Non-diabetic	+ 41.52±18.16 [a]	12.73±1.45	10.35±1.25	ns
Diabetic(STZ)	- 30.26±14.59[b]	22.74±1.12	27.12±2.12	p < 0.001
STZ+Ozone	+ 29.82±6.91[a]	21.47±1.67	16.10±1.45(3)	p < 0.0001
STZ+Oxygen	- 16.27±14.40[b]	21.09±1.94	26.19±1.34(3)	p < 0.01

Data are mean ± SEM; ns: non significant; statistical significance between a and b of at least $p<0.05$.
(1) Changes in corporal weight between the start and the end of the study. Groups with at least a common letter non significant ($p > 0,05$).
(2) After STZ-induced diabetes.
(3) 10 treatments with ozone or oxygen in STZ-induced diabetic rats as described in Methods and Materials.

Ozone treatment increased glutathione (GSH) concentrations with regard to the remaining groups (Table II). The enzymes superoxide dismutase (SOD) and catalase (CAT) showed a similar behavior. Neither GSH nor SOD were different in the remaining groups (non-diabetic rats, STZ-induced diabetes and oxygen-treated diabetic rats). Treatment with O3 caused a reduction in glutathione peroxidase (GPx) with regard to STZ (43%) and STZ + O2 (36%) groups; however, concentrations in ozone-treated diabetic rats were still raised above those seen in non-diabetic control rats.

Table II. Values of glutathione, glutathione peroxidase, catalase and superoxide dismutase in the different experimental groups.

Experimental Groups	GSH μg/g tissue	GPx U/mg protein	CAT U/g protein	SOD U/mg protein
Non-diabetic	602.91 ± 42.12[a]	5.70 ± 0.70[a]	123.34 ± 12.45[a]	2.07 ± 0.17[a]
Diabetic(STZ)[1]	585.14 ± 38.28[a]	21.31 ± 1.21[b]	105.12 ± 8.82[c]	2.13 ± 0.12[a]
STZ+Ozone[2]	702.45 ± 45.43[b]	12.09 ± 0.61[c]	150.03 ± 15.68[b]	2.43 ± 0.11[b]
STZ+Oxygen[2]	576.75 ± 42.61[a]	18.92 ± 1.30[b]	97.06 ± 9.92[c]	2.14 ± 0.16[a]

Data are mean ± SEM. In each column, statistical significance among different letters of at least $p < 0.05$.
(1) After STZ-induced diabetes.
(2) 10 treatments with ozone or oxygen in STZ-induced diabetic rats, as described in Methods and Materials.

Total peroxides were reduced in the O3 group with regard to all treatments, including the control non diabetic. Malondialdehyde concentrations (MDA) were maintained at the level of the control in the treatment with O3 and they diminished, both groups ($p < 0.05$), with relationship to the treatments with STZ and O2 + STZ (Table III).

Table III. Values of total peroxides and malondialdehyde in the different experimental groups.

Experimental Groups	Total Peroxides μmol/g tissue	MDA nmol/mg protein
Non-diabetic	22.11 ± 2.80[a]	0.077 ± 0.006[a]
Diabetic(STZ)[1]	30.53 ± 2.82[c]	0.133 ± 0.008[b]
STZ+Ozone[2]	16.09 ± 2.61[b]	0.068 ± 0.003[a]
STZ+Oxygen[2]	29.74 ± 2.11[c]	0.145 ± 0.009[b]

Data are mean ± SEM. In each column, statistical significance among different letters of at least $p < 0.05$.
(1) After STZ-induced diabetes.
(2) 10 treatments with ozone or oxygen in STZ-induced diabetic rats, as described in Methods and Materials.

Clinical study

In the ozone group, a significant decrease in the glucose figures was achieved (9.1±3.89 vs 6.0±2.61) (Table IV). Also, a significant increase in catalase activity, with a significant decrease of the lipid peroxidation was observed, at the end of the treatment with regard to the initial figures.

Table IV. Antioxidant-Prooxidant balance in patients with microangiopathy complications

Groups	Glucose	GSH	SOD	MDA	CAT
Ozone					
Initial	9.08 ± 33.89	2532 ± 752	59.8 ± 31.3	5.22 ± 1.80	268.3 ± 112
Final	6.03 ±2.69*	2310 ± 877	56.6 ± 33.0	3.12 ± 1.50*	408.3 ±116*
Control					
Initial	9.77 ± 4.16*	2263 ± 903	51.8 ± 21.3	5.52 ± 2.96	183.5 ± 80.0
Final	9.59 ± 3.68	2163 ± 372	47.5 ± 25.3	7.70 ± 4.95	178.1 ± 55.0

Data are mean ± SEM.
* Final vs Initial figures $p < 0.05$

Discussion

Oxidative stress is one of the metabolic events associated to diabetes and its complications (24). It is very important to maintain the antioxidant potential of the pancreatic cell in order to ensure both its survival and insulin secretory capacity during times of increased oxidative stress.

In other studies (12-16) we have demonstrated that using prophylactic ozone, by means of an oxidative preconditioning mechanism, was possible to upregulate the expression of antioxidant enzymes. In this case, we have used ozone after the damage was induced. Our experimental results have shown that ozone diminished the hyperglycemia induced by STZ and also increased antioxidant defenses (Tables I, II, III and IV) either in these conditions.

GSH figures increased significantly, in the ozone group, with respect to the other groups (Table II). The depletion of GSH has been observed so much in experimental diabetes as in clinic. It is reported that hyperglycemia inhibits GSH synthesis, presumably by glycation (25). In type 2 diabetes a decrease of 75 % in GSH figures were observed.

The same behavior as in GSH, an increase in SOD and CAT figures, are observed, for the ozone-treated diabetic rats. Nevertheless, no differences were observed in GSH and SOD figures among the remaining groups (non-diabetic, STZ-induced diabetes and oxygen-treated diabetic groups). This behavior may be due to compensating mechanisms similar to that one which was found for (mRNA) SOD in STZ-treated rats (26). CAT figures were much lower in STZ-induced diabetes and oxygen treated diabetes with respect to non diabetic group. Our data is in coincidence with previous reports (27-29) that have shown increased activities of SOD, catalase and peroxidases after chronic O3 exposure. On the other hand in aorta from diabetic rats in vitro, addition of SOD to the bathing medium partially improved defective acetylcholine-stimulated relaxation (30). The resultant reduction in GSH concentrations, which also leads to SOD downregulation (31) would compromise ROS protection.

NADPH is a co-factor for the reduction of oxidized glutathione (GSSG), which is important for the glutathione peroxidase-catalyzed elimination of peroxides. H_2O_2, scavenged by

catalase and GPx, has been associated to tissue damage in diabetes (32). The ability of glomeruli isolated from STZ-induced diabetic rats to degrade H_2O_2 was greatly impaired; this has been attributed to either a decreased CAT activity or an altered GSH redox cycle (33). Also, it has been demonstrated a role of H_2O_2 in proteins cross-linking in diabetes (34). Ozone increased the GPx figure in comparison with the non diabetic group, but lower with respect to STZ and STZ+O2 groups. Ozone treatment stimulated or preserved mechanisms of antioxidant defenses. It reduced the levels of total peroxides with regards to the remaining groups and it maintained the concentrations of MDA (Table III) at the level of the non diabetic group. MDA and the peroxides have been associated to diabetes and their complications, achieving in this study high figures in STZ and STZ+O2 groups. The increase observed in GPx, in these 2 groups (STZ and STZ+O2), was not enough to upregulate the high figures of MDA and total peroxides obtained. An approximately three-fold increase in ROS production accompanied by a similar elevation of malondialdehyde, an index of lipid peroxidation, was seen in rat aorta after 1 month of diabetes (35).

Antioxidant-prooxidant balance, associated to the control of oxidative stress was favored by ozone treatment, while the group treated with oxygen did not differ of the STZ-induced diabetic rats.

In patients, in the ozone group, a significant decrease in the glucose figures, with a significant increase in catalase activity and a significant decrease of the lipid peroxidation were observed, at the end of the treatment with regard to the initial figures. In the group treated with antibiotics, no change was achieved in the studied parameters. It is known (36) that diabetic patients have lowered antioxidant defenses, both enzymatic (SOD, CAT, glutathione peroxidase) and non-enzymatic (GSH) with an increase oxidative damage. Polymorphonuclear cells of both type 1 and type 2 diabetic showed approximately twofold diminution of SOD activity. The same trend was found in lymphocytes but marginally greater in the type 2 group (37). Superoxide anion is dismutated by SOD to hydrogen peroxide (H_2O_2), that is a powerful oxidant, associated to tissue damage in diabetes (32). Catalase is a scavenger of H_2O_2. The increased in catalase activity observed in the group treated with ozone suggests that ozone promoted the captured of H_2O_2, a precursor of hydroxil radical, being the last one, capable of producing the peroxidation of unsaturated fatty acids, with the damage of the cell membrane functions. Also, MDA have been associated to diabetes and their complications (36). Ozone was capable to produce a significant decrease of this parameter. These results suggest that ozone protective effects on antioxidant endogenous defense improve glucose metabolism.

Clinically, patients treated with ozone therapy had a better and faster recovery (15 days vs 21 days) of their lesions in comparison with the patients treated with antibiotic therapy. Any side effect was found.

Conclusions

In summary, the ozone treatment, in both studies, improved glycemic control and prevented oxidative stress associated to diabetes mellitus and its complications, in agreement with the excellent results obtained clinically in these patients.

Keywords

Ozone; streptozotocin-induced diabetes; diabetic foot; oxidative stress; antioxidant defense system.

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