Ozone treatment reduces markers of oxidative and endothelial damage in an experimental diabetes model in rats

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Abstract

***Background*:** Diabetes produces a large number of changes in vessels that affect the reactivity of smooth muscle and endothelium. Vascular endothelium appears to be a vulnerable target for hyperglycemia-induced metabolic changes. Activation of polyol pathway, non-enzymatic glycosilation of proteins and the increase of reactive oxygen species (ROS) play an important role in diabetes complications. Ozone has been used as a therapeutical agent and beneficial effects have been observed. However, so far only a few biochemical and pharmacodynamic mechanisms have been elucidated. We demonstrated that controlled ozone administration may promote an oxidative preconditioning or adaptation to oxidative stress, preventing the damage induced by reactive oxygen species (ROS).

Aim: Taking into account that diabetes is a disorder associated with oxidative stress, we postulate that ozone treatment might protect antioxidant systems and maintain, at a physiological level, other markers of endothelial cell damage associated with diabetic complications.

Methods: Five groups of rats were classified as follows: (1) control group treated only with physiological saline solution; (2) positive control group using streptozotocin (STZ) as a diabetes inductor; (3) ozone group, receiving 10 treatments (1.1 mg / kg), one per day after STZ–induced diabetes; (4) oxygen group (26 mg /kg), one per day, as in group 3, but using oxygen only; (5) control ozone group, as in group 3, but without STZ. The following parameters were evaluated: plasma glucose concentration; in pancreas homogenates: levels of aldose reductase, fructolysine, advanced oxidation protein products, nitrite/nitrates (as an index of nitric oxide), glutathione (GSH), total hydroperoxides (TH); MDA concentration; catalase (CAT); superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities as indicators of redox balance. Pancreas morphology was evaluated by light microscopy.

Results: Ozone treatment improved glycemic control, pancreas integrity and prevented oxidative stress, the increase of aldose reductase, fructolysine content and advanced oxidation protein products. Nitrite and nitrate levels were maintained without changes with regard to non-diabetic control.

Conclusions: The results of this study show that repeated administration of ozone in non-toxic doses might play a role in the control of diabetes and its complications.

Introduction

Diabetes produces a large number of changes in vessels that affect the reactivity of smooth muscle and endothelium, the production of vasoactive substances by endothelium, vessel wall permeability to macromolecules, susceptibility to atherosclerosis and activity of the thrombolytic system [1-3].

These events are related to the chronic vascular complications of this disorder. The vascular lesion in diabetes consists of microangiopathy, distinguished by thickening of capillary basement membranes resulting in increased vascular permeability. These changes are clinically manifested as diabetic retinopathy and/or microangiopathy, which consists of atheromatous involvement of large blood vessels. Macroangiopathy is morphologically similar to non-diabetic atheroma, but rending to occur earlier and be more extensive [4].

Vascular endothelium appears to be vulnerable target for hyperglycemia-induced metabolic changes. High glucose concentrations promote endothelial cells damage by different mechanisms probably through mutual facilitatory interactions between them [5]. Activation of polyol pathway, non-enzymatic glycosylation of proteins and the increase of Reactive Oxygen Species (ROS) play an important role in diabetes complications.

Ozone administered by rectal insufflation in dose and number of controlled treatments, has shown protective effects against the damage induced by carbon tetrachloride and hepatic and renal ischemia-reperfusion through a probable mechanism of oxidative preconditioning which confer protection by stimulation of antioxidant endogenous systems, accumulation of adenosine and by blocking the xanthine/xanthine oxidase pathway for ROS generation [6-9]. In addition, it has been demonstrated the decrease of blood cholesterol and stimulation of antioxidative response in cardiopathy patients treated with intravenous ozone therapy [10].

Materials and Methods

Animals

Male Sprague-Dawley rats weighing 250-278 g were obtained from CENPALAB (Bejucal, Havana, Cuba). Animals were housed in temperature and light-controlled rooms and allowed free access to normal diet pellet and tap water. All procedures were performed as approved by the Institutional Animal Care Committees (ARCA No.012) 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 [11]. 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 [12]. Blood glucose was measured using a diagnostic kit obtained from Sigma 315-100 (Sigma, St. Louis, MO, USA) based on a colorimetric reaction.

Animals and Treatment

The protocol consisted of five experimental groups (n =10, 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}, a dose of ozone in which the phenomenon of oxidative preconditioning is achieved without appreciable toxicity [6-9]) one per day after STZ-induced diabetes; (4) Oxygen, vehicle of O_3 (26 mg kg^{-1}, dose equivalent to the O_2 concentration present in one O_3 dose) one per day, as in group 3 but using oxygen only; (5) Control ozone, as group 3, but without STZ. The ozone concentration in the O_3/O_2 mixture was 50 μg mL^{-1}.

Ozone was generated by an OZOMED equipment manufactured by the Ozone Research Center (Cuba) and was administered by rectal insufflation. Ozone was obtained from medical grade oxygen, was used immediately as generated and it represented only about 3% of the gas (O_2 + O_3) mixture. The ozone concentration is measured by using a build-in UV spectrophotometer set at 254 nm (accuracy, 0.002 A at 1A, repeatability 0.001 A and calibrated with internal standard). The ozone dose is the product of the ozone concentration (expressed as mg L^{-1} by the gas (O_2 + O_3)

volume (L). By knowing the body weight of the rat the ozone dose is calculated as mg kg^{-1} as in our previous papers [6-9].

After 11 days of diabetic induction, blood glucose was measured, body weight of the animals was monitored and then they were euthanized by diethyl ether anesthesia. Afterwards pancreas was promptly removed for biochemical and histological studies. Pancreas homogenates were obtained using a tissue homogenizer Edmund Bühler at 4°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°C and 8500 x g during 20 min. Supernatants were taken for biochemical determinations.

Biochemical determinations
The biochemical parameters were evaluated in the supernatants of pancreas homogenates 11 days after STZ-induced diabetes and 24 hours after the last treatment with ozone or oxygen, as it corresponded.

The different 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 [13]. Superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activity 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°C was measured at a wavelength of 586 nm. For standards, freshly prepared solutions of malondialdehyde bis [dimethyl acetal] (Sigma St. Louis, MO, USA) were employed and assayed under identical conditions [14]. Quantification of total hydroperoxides was measured by Bioxytech H_2O_2-560 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 [15]. After precipitation of thiol proteins using TCA 10%, the reduced glutathione (GSH) were measured according to the method of Sedlak and Lindsay [16] with the Ellman's reagent (5,5' dithiobis (2-nitrobenzoic acid) 10^{-2}M (Sigma St. Louis, MO, USA), the absorbance was measured at 412 nm . Nitrite/nitrate levels were determined by the Griess reaction by first converting nitrates to nitrites using nitrate reductase (Boehringer Mannheim Italy SpA, Milan, Italy). Then the Griess reagent (1% sulphanilamide, 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride in 0.25 % phosphoric acid was added [17]. Samples were incubated at room temperature for 10 min and absorbance was measured at 540 nm using a microplate reader. The advanced oxidation protein products (AOPP) were measured through the oxidation of iodide anion to diatomic iodine by AOPP [18]. Relative fructolysine content (Amadori's product of glycated serum protein) was measured by reduction by the redox indicator nitrobluetetrazolium (NBT) at 530 nm [19]. Aldose reductase activity was determined using a conventional procedure [20].

Statistical analysis
The OUTLIERS preliminary test for detection of error values was initially applied for statistical analysis. Afterward, the ANOVA method (single way) was used followed by homogeneity variance test (Bartlett-Box). In addition, a multiple comparison test was used (Duncan test). Data were expressed as the mean ± standard deviation of 10 animals. The level of statistical significance employed was at least $p<0.05$ for all experiments.

Results and Discussion

Body weights and blood analysis
Rats treated with streptozotocin (STZ) and STZ + O_2 were hyperglycemic and lost weight over the experimental period (table 1). 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 1. Body weight and plasma glucose concentrations

Groups	n	Body weight changes (g)[1]	Plasma (mmol Glucose l^{-1})		Statistical significance of plasma glucose
			Start	End[2]	
Non-diabetic control	10	+41.52±18.16[a]	12.73±1.45	10.35±1.25	ns
Diabetics (STZ)	10	-30±14.59[b]	22.74±1.12	27.12±2.12	P < 0.001
STZ+Ozone	10	+29.82±6.91[a]	21.47±1.67	16.1±1.45[3]	P < 0.0001
STZ+Oxygen	10	-16.27±14.40[b]	21.09±1.94	26.19±1.34[3]	P < 0.01
Ozone	10	+38.20±16.15[a]	11.20±1.16	11.50±1.18	ns

Note: Date are mean ± SEM. ns: non-significant. (1) Changes in corporal weight between the start and end of the experiment. Groups with at least a common letter as superscript non-significant (P>0.05). (2) 10 days after STZ-induced diabetes. (3) after 10 treatments with ozone or oxygen in STZ-induced diabetic rats.

Antioxidant-prooxidant balance

The O_3 + STZ treatment increased glutathione (GSH) concentrations with regard to the remaining groups (Fig. 1A). The enzymes superoxide dismutase (SOD) and catalase (CAT) showed a similar trend(Fig. 1 B, C). Neither GSH nor SOD were different in the remaining groups (non-diabetic, STZ-induced diabetes, O_2-treated diabetic or O_3-treated rats). Treatment with ozone caused a reduction in glutathione peroxidase with regard to STZ (43%) and STZ + O_2 (36%) groups; however, concentrations in ozone-treated diabetic rats were still raised above those seen in non-diabetic control rats (Fig. 1D). Total peroxides were reduced in the ozone-treated group with regard to all treatments, including the control non diabetic (Fig. 2A), whereas malondialdehyde (MDA) concentrations were maintained at the level of the control in the animals treated with O_3 or in the group treated with O_3 + STZ ($p<0.05$) and a significant increase was noted in the treatments with STZ and O_2 + STZ ($p<0.05$) with respect to control group.

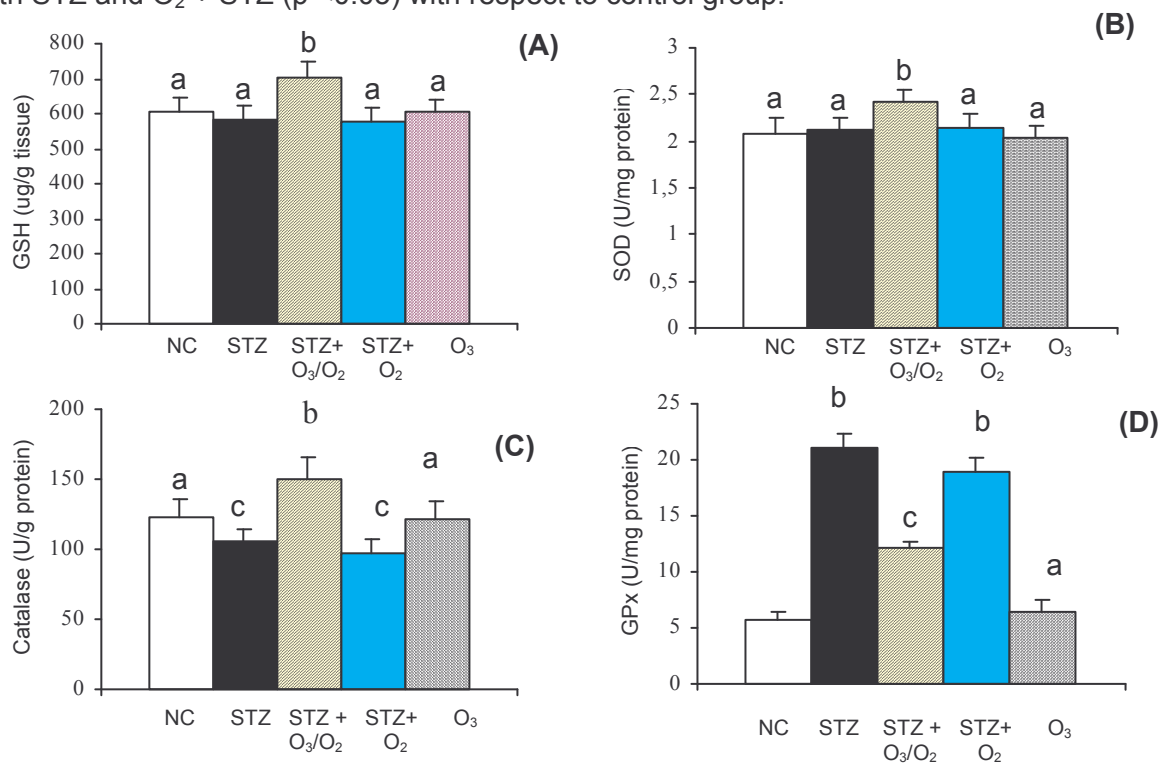

Fig. 1. *Behavior of antioxidant systems in non-diabetic and diabetic rats: NC, non-diabetic controls; STZ, diabetic group induced by streptozotocin 45 mg kg^{-1} i.p.; STZ + O_3/O_2, diabetic group treated with ozone (1.1 mg kg^{-1}) 10 treatments by rectal insufflation; STZ + O_2, diabetic group treated with oxygen, vehicle of ozone (26 mg kg^{-1}) 10 treatments by rectal insufflation. Data are means ± SEM. Means having different superscript letters indicate significant difference ($p<0.05$) between groups.* ***(A)****: Glutathione (GSH);* ***(B)****: Superoxide dismutase (SOD);* ***(C)****: Catalase (CAT);* ***(D)****: Glutathione Peroxidase (GSH-Px).*

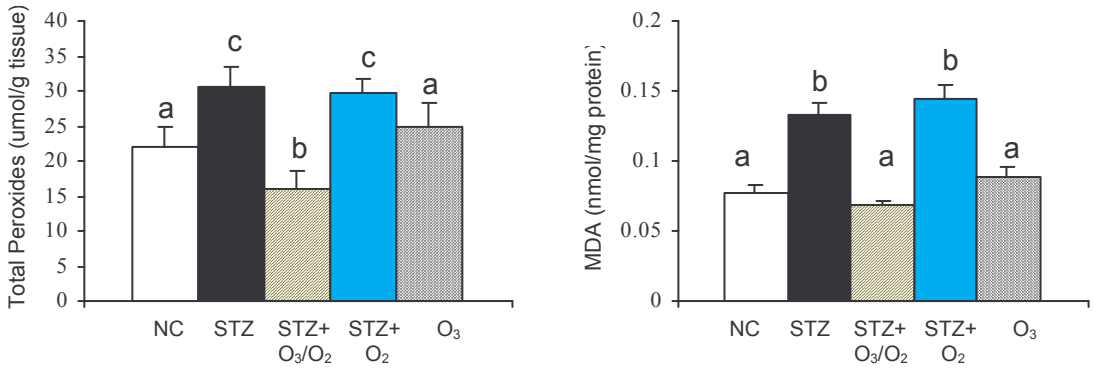

Fig. 2. *Levels of total hydroperoxides* ***(A)*** *and lipid peroxidation products* ***(B)*** *in non-diabetic and diabetic rats. NC, non-diabetic; STZ, diabetic group induced by streptozotocin 45 mg kg^{-1} i.p.; STZ + O_3/O_2, diabetic group treated with ozone (1.1 mg kg^{-1}) 10 treatments by rectal insufflation; STZ + O_2, diabetic group treated with oxygen, vehicle of ozone (26 mg kg^{-1}) 10 treatments by rectal insufflation. Data are means ± SEM. Means having different superscript letters indicate significant difference ($p<0.05$) between groups.*

Histological studies showed the protective effect on α and β cells by ozone in STZ-induced diabetes (Fig. 3).

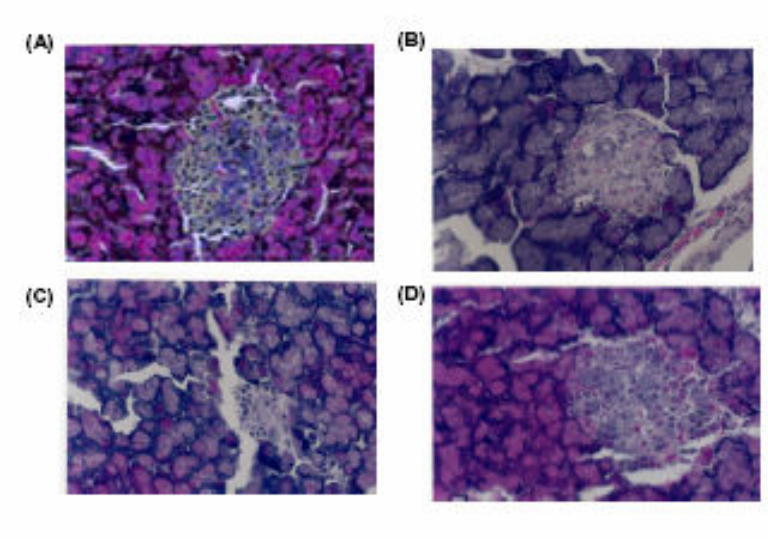

Fig. 3. Histological results in non-diabetic and diabetic rats: (A) Non-diabetic control group. Normal appearance of β-cells (dark blue) and α-cells (red); (B) STZ-induced diabetes. Loose of β-cells and cellular vacuolization; (C) STZ-induced diabetes + Oxygen. Severe damage with atrophic of pancreatic islets; (D) STZ-induced diabetes + Ozone. Normal aspect of the islets (β-cells, blue; α-cells, red). Gomori, 400 x.

Biomarkers of the polyol pathway, non-enzymatic glycosylation, protein oxidation and nitric oxide. The results obtained for these parameters are shown in the table 2.

Aldose reductase activity which catalyze the reduction of glucose to sorbitol and the relative fructolysine content, precursor of Advanced Glycation Endproducts (AGEs) was significantly ($p<0.05$) increase in STZ and O_2-STZ diabetic rats. On the other hand, there was no significant differences when compared the diabetic rats treated with ozone and the control non diabetic. Ozone group did not significantly ($p<0.05$) modify the aldose reductase activity with regard to normal control rats. It was found a close relation ($r = 0.78$, $p<0.05$) between relative fructolysine content and Advanced Oxidation Protein Products (AOPP) concentrations.
The levels of NO_2/NO_3, in the ozone-treated group, did not differ from the control group. Both groups showed significantly higher concentrations with regard to STZ and STZ + O_2.

Table 2. Levels of aldose reductase, fructolysine, advanced oxidation protein products and NO_2/NO_3

Groups	AR[(1)]	FA[(2)]	AOPP[(3)]	NO_2/NO_3[(4)]
Non-Diabetic Control	0.58±0.09[a]	6.36±0.00[a]	38.53±1.67[a]	11.74±0.74[a]
Diabetic (STZ)	1.38±0.08[b]	32.13±0.87[b]	44.14±1.24[c]	5.32±0.98[b]
STZ+Ozone	0.58±0.10[a]	9.36±0.01[a]	31.09±1.39[b]	12.61±0.82[a]
STZ+Oxygen	1.17±0.08[b]	38.98±0.63[b]	43.48±1.30[c]	6.21±1.27[b]
Ozone	0.56±0.05[a]	5.96±0.02[a]	35.42±1.5[a]	11.3±0.6[a]

Legend: (1) AR: aldose reductase (mmol glucose /min/ mg protein, (2) FA: fructolysine (relative fructolysine content/ mg protein x 10^{-5}, (3) AOPP: advanced oxidation protein products (μmol chloramine-T equivalent /mg protein, (4) NO_2/NO_3: nitrites/nitrates (mmol /mg protein). Date are mean ± SEM. Means having different superscript letters indicate significant differences ($P<0.05$) between groups.

Most previous studies have focused on immediate or concurrent factors, which contribute to the phenomenon of diabetes-induced endothelial dysfunction. In the present study we have integrated some of the most important metabolic events associated to the diabetic endotheliopathy process and its control by ozone treatment.

It is of critical importance 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. On the other hand, pancreas is the main target of STZ.

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

Ozone reduced STZ-induced hyperglycemia and it increased antioxidant defenses (GSH, SOD and CAT levels) of pancreas (Fig.1. A,B,C). The capacity of ozone to enhance antioxidant endogenous systems, in front of oxidative stress by oxidative preconditioning or adaptative mechanisms has been demonstrated [6].

There is evidence that hyperglycemia can lower both the activity of a number of enzymes including SOD [21] and GSH synthesis, presumably by glycation [22]. It is not possible at this knowledge state to define how ozone treatment decrease the hyperglycemia. However the observation that diabetic patients have lowered antioxidant defenses, both enzymatic (SOD, CAT, GSH-Px) and non-enzymatic (vitamin C, E or A, free radical scavengers or 'total radical-trapping antioxidant capacity') is almost as well established as the observation of increased oxidative damage [21]. Therefore, these results suggest that ozone protective effects on antioxidant endogenous defenses improve the glucose metabolism.

In line with the increase in antioxidant systems there was a reduction of total peroxides and the concentrations of MDA were at the level of the control group(fig. 2). MDA and peroxides have been associated to diabetes and its complications. An approximately three-fold increase in ROS production accompanied by a similar elevation of MDA, an index of lipid peroxidation, was seen in rat aorta after 1 month of diabetes [23]. In addition, it has been demonstrated a role of H_2O_2 in proteins cross-linking in diabetes [24].

No differences were observed in GSH and SOD among 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 [24].

As it was analyzed, the treatment with ozone maintained the necessary antioxidant-prooxidant balance. Nevertheless, endothelium integrity and function depend not only on the ROS control but also of possible modes of action and some potential interactions between the polyol pathway, ROS production, advanced glycation endproducts and NO generation [5, 25, 26].

The concentrations of the mediators derived from the increased flux of glucose through the polyol pathway (aldose reductase and fructolysine) were reduced by ozone treatment while Advanced Oxidation Protein Products (AOPP) were not increased in ozone treatment group. In correspondence with these results, it was found a close relation between fructolysine contents and AOPP concentrations ($r = 0.78$, $p < 0.05$).

The regulative effects of ozone on aldose reductase activity represent another interesting action of this complementary medical approach since aldose reductase is a key enzyme of the polyol pathway and their inhibitors have been used as therapeutical drugs linked to improve NO production or release [27] , through NADPH-sparing activity that helps to replenish antioxidants reserves, thus having an indirect antioxidant action and in mild diabetic neuropathy or in preventing periphereal and autonomic neuropathy in unaffected unaffected diabetic patients [28] .

Substancial evidence exists that diabetes results in impaired endothelial dysfunction suggesting diminished nitric oxide production from diabetic endothelium [29].

Ozone treatment prevented depletion of NO_2/NO_3 (table 2). This result indicate that NO production has not been affected by STZ-induced diabetes. Thus, ozone may protect against the imbalance in NO-ROS interactions, improve NO-mediated relaxation and decrease microvessels reactivity, in this experimental model of diabetes.

Conclusion

In summary, ozone treatment improved glycemic control, prevented oxidative stress, the increase of aldose reductase, fructolysine content and advanced oxidation protein products. NO_2/NO_3 levels were maintained without changes with regard to non-diabetic control. These events are closely related with endothelial damage, therefore these results suggest that ozone, in our experimental conditions, may have a role in the treatment of diabetic complications. Other works studying the effects of ozone on diabetic patients with macroangiophatic complications are in progress.

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