Pharmacological Research, Vol. 44, No. 5, 2001
doi:10.1006/phrs.2001.0867, available online at http://www.idealibrary.com on IDEAL®

OZONE TREATMENT REDUCES MARKERS OF OXIDATIVE AND ENDOTHELIAL DAMAGE IN AN EXPERIMENTAL DIABETES MODEL IN RATS

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Accepted 17 July 2001

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 demonstrate that controlled ozone administration may promote an oxidative preconditioning or adaptation to oxidative stress, preventing the damage induced by reactive oxygen species (ROS). Taking into account that diabetes is a disorder associated with oxidative stress, we postulate that ozone treatment in our experimental conditions might protect antioxidant systems and maintain, at a physiological level, other markers of endothelial cell damage associated with diabetic complications. 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^{-1}), one per day after STZ-induced diabetes; (4) oxygen group (26 mg kg^{-1}), one per day, as in group 3 but using oxygen only; (5) control ozone group, as group 3, but without STZ. The ozone treatment improved glycemic control 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. 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.

KEY WORDS: ozone, streptozotocin-induced diabetes, oxidative stress.

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 tending to occur earlier and be more extensive [4].

Vascular endothelium appears to be a vulnerable target for hyperglycemia-induced metabolic changes. High glucose concentrations promote endothelial cell 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 diabetic complications.

Ozone, administered by rectal insufflation in a 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 confers 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, a decrease of blood

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1043–6618/01/110391–06/$35.00/0

cholesterol and stimulation of antioxidative response in cardiopathy patients treated with intravenous ozone therapy has been demonstrated [10].

Taking the view that diabetes promotes an oxidative damage and ozone protects the cells in oxidative stress situations, we investigated the actions of ozone on streptozotocin-induced diabetes, characterizing the redox balance and its relation with markers of polyol pathway, non-enzymatic glycosylation of proteins and the levels of nitrates and nitrites, as a measure of nitric oxide (NO) production.

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 pellets 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 h 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 group, 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 group, 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 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 upon generation and represented only about 3% of the gas $(O_2 + O_3)$ mixture. The ozone concentration is measured by using a built-in UV spectrophotometer set at 254 nm (accuracy, 0.002 Å at 1 Å, repeatability 0.001 Å 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, the body weight of the animals was monitored and then they were killed by diethyl ether anesthesia. Afterwards the pancreas was promptly removed for biochemical 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 ***g*** for 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 h after the last treatment with ozone or oxygen, respectively.

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 s intervals for 1 min [13]. Superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) 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,USA). 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) was measured according to the method of Sedlak and Lindsay [16] with 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

Table I
Body weight and plasma glucose concentrations

Groups	*n*	*Body weight changes (g)*(1)	*Plasma glucose (mmol l^{-1})*		*Statistical significance of plasma glucose*
			Start	*End*(2)	
Non-diabetic control	10	$+41.52 \pm 18.16^a$	12.73 ± 1.45	10.35 ± 1.25	ns
Diabetic(STZ)	10	-30.26 ± 14.59^b	22.74 ± 1.12	27.12 ± 2.12	$P < 0.001$
STZ + Ozone	10	$+29.82 \pm 6.91^a$	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	$+38.20 \pm 16.15^a$	11.20 ± 1.16	11.50 ± 1.18	ns

Data 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 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 as described in Materials and Methods.

Table II
Levels of aldose reductase, fructolysine, advanced oxidation protein products and NO_2NO_3

Groups	*AR*(1)	*FA*(2)	*AOPP*(3)	NO_2/NO_3(4)
Non-diabetic control (NC)	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

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

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 of 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 the 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

Body weights and blood analysis

Rats treated with streptozotocin (STZ) and STZ + O_2 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 for the non-diabetic control.

Antioxidant–prooxidant balance

The O_3 + STZ treatment increased glutathione (GSH) concentrations with regard to the remaining groups [Fig. 1(a)]. 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. 1(d)]. Total peroxides were reduced in the ozone-treated group with regard to all treatments, including the control non-diabetic [Fig. 2(a)], 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.

Biomarkers of the polyol pathway, non-enzymatic glycosylation, protein oxidation and nitric oxide

The results obtained for these parameters are shown in Table II. Aldose reductase activity which catalyzes the reduction of glucose to sorbitol and the relative fructolysine content, precursor of Advanced Glycation Endproducts (AGEs) was significantly ($P < 0.05$) increased in STZ and O_2-STZ diabetic rats. On the other hand, there was no significant differences when comparing the diabetic rats treated with ozone and

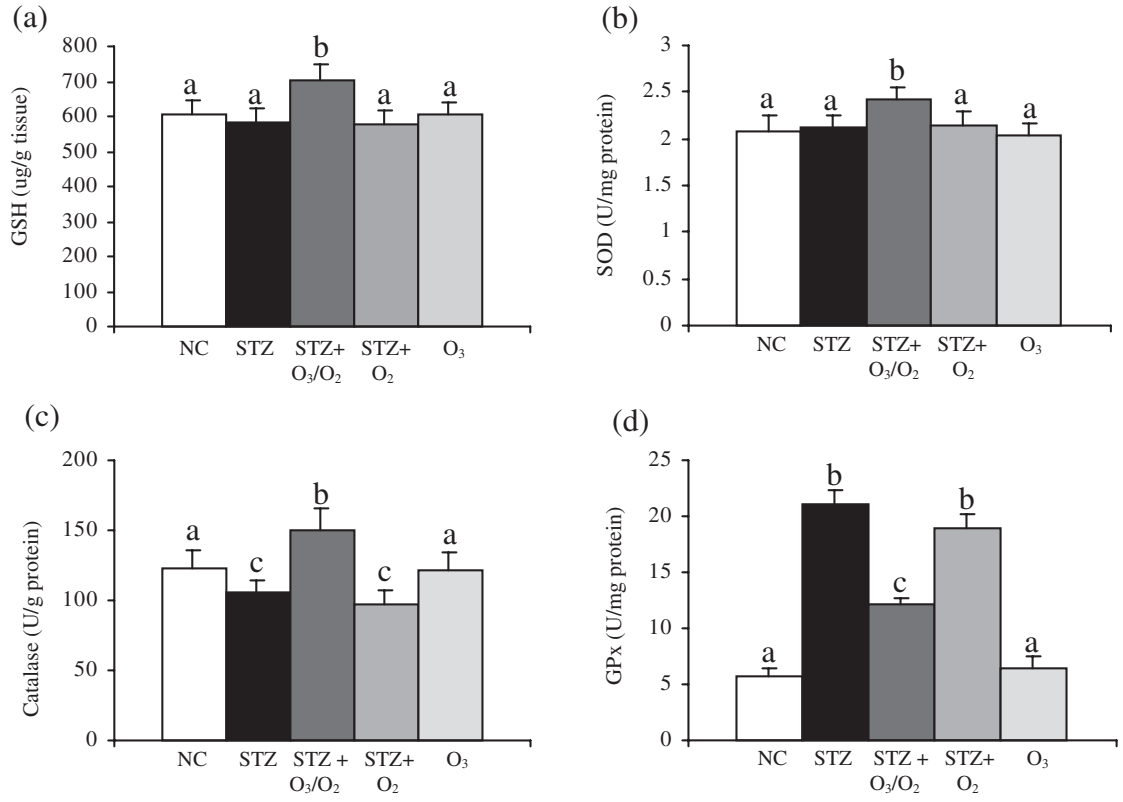

Fig. 1. Behavior of antioxidant systems in non-diabetic and diabetic rats: NC, non-diabetic controls; STZ, diabetic group induced by streptozotocin 45 mg kg^{-1} i.p.; STZ + O_3/O_2, diabetic group treated with ozone (1.1 mg kg^{-1}), 10 treatments by rectal insufflation; STZ + O_2, diabetic group treated 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).

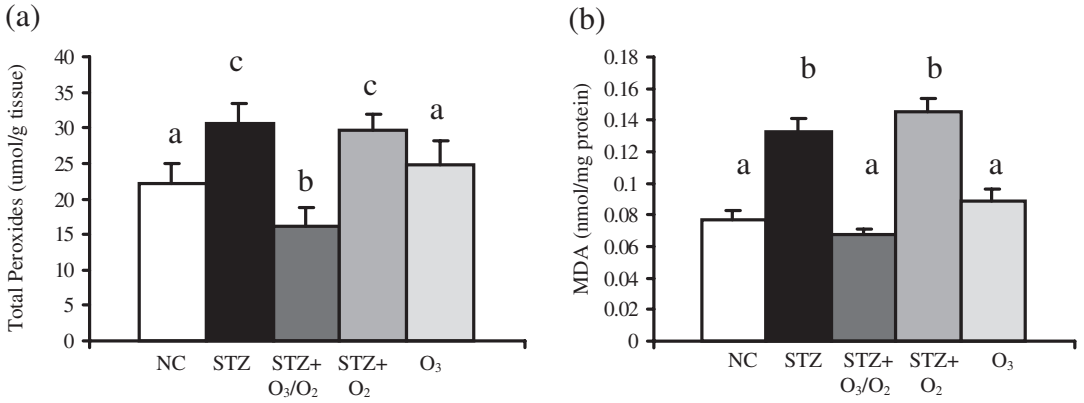

Fig. 2. Levels of total hydroperoxides (a) and lipid peroxidation products (b) in non-diabetic and diabetic rats. NC, non-diabetic; STZ, diabetic group induced by streptozotocin 45 mg kg^{-1} i.p.; STZ + O_3/O_2, diabetic group treated with ozone (1.1 mg kg^{-1}), 10 treatments by rectal insufflation; 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.

the control non-diabetic. The ozone group did not significantly ($P < 0.05$) modify the aldose reductase activity with regard to normal control rats. A close relation was found ($r = 0.78$, $P < 0.05$) between relative fructolysine content and 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.

DISCUSSION

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 with 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, the pancreas is the main target of STZ.

The antioxidant–prooxidant balance, associated with 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 the antioxidant defenses (GSH, SOD and CAT levels) of the 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 decreases 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 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 with 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, a role for H_2O_2 has been demonstrated in protein 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 the one which was found for (mRNA) SOD in STZ-treated rats [24].

When 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 on 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 AOPP were not increased in the ozone treatment group. Corresponding with these results, a close relation between fructolysine contents and AOPP concentrations was found ($r = 0.78$, $P < 0.05$).

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 its inhibitors have been used as therapeutical drugs linked to improving NO production or release [27]. This is brought about through NADPH-sparing activity that helps to replenish antioxidant reserves, thus having an indirect antioxidant action in mild diabetic neuropathy or in preventing periphereal and autonomic neuropathy in unaffected diabetic patients [28].

Substantial 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 II). This result indicates 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 microvessel reactivity, in this experimental model of diabetes.

In summary, ozone treatment improved glycemic control and 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.

ACKNOWLEDGEMENTS

These studies were supported in part by Randox Laboratories (Antrim, UK) and the Department of Chemistry and Medical Biochemistry, University of Milan.

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