Therapeutic efficacy of ozone in patients with diabetic foot

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Abstract

Oxidative stress is suggested to have an important role in the development of complications in diabetes. Because ozone therapy can activate the antioxidant system, influencing the level of glycemia and some markers of endothelial cell damage, the aim of this study was to investigate the therapeutic efficacy of ozone in the treatment of patients with type 2 diabetes and diabetic feet and to compare ozone with antibiotic therapy. A randomized controlled clinical trial was performed with 101 patients divided into two groups: one ($n=52$) treated with ozone (local and rectal insufflation of the gas) and the other ($n=49$) treated with topical and systemic antibiotics. The efficacy of the treatments was evaluated by comparing the glycemic index, the area and perimeter of the lesions and biochemical markers of oxidative stress and endothelial damage in both groups after 20 days of treatment. Ozone treatment improved glycemic control, prevented oxidative stress, normalized levels of organic peroxides, and activated superoxide dismutase. The pharmacodynamic effect of ozone in the treatment of patients with neuroinfectious diabetic foot can be ascribed to the possibility of it being a superoxide scavenger. Superoxide is considered a link between the four metabolic routes associated with diabetes pathology and its complications. Furthermore, the healing of the lesions improved, resulting in fewer amputations than in control group. There were no side effects. These results show that medical ozone treatment could be an alternative therapy in the treatment of diabetes and its complications.
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1. Introduction

Diabetes mellitus is characterized by metabolic abnormalities. It is a disorder of carbohydrate metabolism resulting in hyperglycemia and glycosuria, due to an inadequate production or utilization of insulin. Long-term complications causing morbidity and premature mortality are characterized by microvascular disease with capillary basement membrane thickening, macrovascular disease with accelerated arterio-Hong sclerosis, neuropathy involving both the somatic and autonomic nervous systems, neuromuscular dysfunction with muscle wasting, embryopathy, and decreased resistance to infections. Such chronic complications involve the eyes, kidneys, heart, nerves, and blood vessels. Accelerated arteriosclerosis accounts for 80% of diabetic mortality, three fourths of it due to coronary disease. A more frequent concomitant of distal anesthesia is the development of neurotropic ulceration, particularly on the plantar side of the foot. Anesthesia leads to worsening of any minor injury because of the absence of protective painful stimuli. A loss of sensation is the major stumbling block to early problem recognition. These changes combined with the pre-existing microvascular and macrovascular circulatory impairments characterize the mechanisms that may lead to gangrene after foot injury (Stein, 1994; Fauci et al., 1998).

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In diabetic patients the role of reactive oxygen species, leading to increased oxidative damage at the level of lipid peroxidation with DNA injury and protein damage, has been demonstrated (Cameron and Cotter, 1994; Halliwell and Cross, 1994; Sinclair and Lunce, 1995; Leinomen et al., 1997; Schleicher et al., 1997). Four main molecular mechanisms are involved in glucose-mediated vascular damage: increased polyol pathway flux; increased advanced glycation end-product formation; activation of protein kinase C (PKC) isoforms; and increased hexosamine pathway flux (Skiha et al., 1996). A hyperglycemia-induced process of overproduction of superoxide by the mitochondrial electron-transport chain seems to occur. More factors have been also considered, such as a decrease in the antioxidant defence system, involving erythrocyte superoxide dismutase and catalase (Akkus et al., 1996; Atalay et al., 1997), with a simultaneous decrease in vitamin C concentration in leukocytes and a decrease in the scavenger capacity of radicals in plasma (Ceriello et al., 1997).

Ozone can exert protective effects by oxidative preconditioning, stimulating and/or preserving the endogenous antioxidant systems and by blocking the xanthine/xanthine oxidase pathway for reactive oxygen species generation, as demonstrated in studies of the damage induced by carbon tetrachloride (CCl_4) or by hepatic and renal ischemia–reperfusion (León et al., 1998; Barber et al., 1999; Peralta et al., 1999, 2000). Moreover, ozone oxidative preconditioning has been shown to preserve glycogen content and to reduce lactate and uric acid formation, controlling oxidative stress induced by CCl_4 administration in rats (Candelario-Jalil et al., 2001). In addition, endovenous ozone therapy in patients with myocardial infarction has been shown to have a beneficial effect on blood lipid metabolism by reducing the blood cholesterol concentration and activating the antioxidant protection system (Hernández et al., 1995). Ozone has been used with good results in the treatment of patients with diabetic foot, because of its germicidal properties and its influence on the processes of oxygen metabolism, and other effects (Velasco et al., 1989).

Some epidemiological studies suggest that there will be 300 million diabetic patients with mainly type 2 diabetes by 2010 (Rösen et al., 2001). Approximately 15% of people with diabetes will develop a foot ulcer in their lifetime, at a rate of 2% to 3% a year. More than 50% of non-trauma-related lower limb amputations in the United States occur in people with diabetes. Of these amputations, 25% are below the knee and 20% above. The 3-year mortality after amputation is 20–50% and 5-year mortality is 39–68% (Reiber et al., 1995). Therefore, the socio-economic impact of diabetes is devastating both to the individual and to society. Any treatment capable of stabilizing oxygen metabolism and modulating oxidative stress, accompanied by germicidal actions, can improve the quality of life of these patients and reduce their medicine use. Given the therapeutic properties of ozone, the aim of this study was to evaluate the effectiveness of ozone in the treatment of patients with type 2 diabetes suffering from diabetic foot complications, and its effects on oxidative stress, hyperglycemia and some markers of endothelial damage, to compare ozone with antibiotic therapy.

2. Materials and methods

2.1. Study design

This randomized controlled clinical trial was approved by an institutional review board (Scientific and Ethics Committees of the Institution) in accordance with the principle of the Declaration of Helsinki (1997). All patients gave their informed consent to being enrolled after receiving adequate information about the study (characteristics of the study, benefits and possible side effects). Before enrolling, all participants attended a training program to familiarize them with the study objectives and treatment plans. The personnel involved emphasized that all participating physicians would treat each patient according to the randomised scheme of treatment.

Adult patients of both sexes and different ethnic origins with a diagnosis of neuroinfectious diabetes foot, according to the classification by McCook et al. (1971), were suffering from ulcer of the feet and lower extremities, and who were hospitalized in the Institute of Angiology and Vascular Surgery (La Habana, Cuba) were eligible to participate in the study. Exclusion criteria were: severe septic conditions, hypersensitivity to the medication to be used, hepatic dysfunction, renal failure (serum creatinine level >1.32 μmol/l), pregnancy, cancer or other serious disease, inability to cooperate with the requirements of the study, recent history of alcohol or drug abuse, current therapy with any immunosuppressive agent or anticonvulsant, concurrent participation in another clinical study, or current treatment with an investigational drug.

For the calculation of the size of the sample, the MEDSTAT system (version 2.1, 1989) was used. The type 1 error was 5% and the type 2 error was 20%, with a minimal difference between effect rates not higher than 25%. The target level of enrolment was determined to be 43 patients per group. Assuming that 10% of study patients would be lost to follow-up, 100 patients were studied.

Patients were randomized to two different groups of treatment: 1) antibiotic therapy; 49 patients were treated with systemic antibiotic therapy (according to the microbe present), using the conventional method of treatment, with topical application to the lesion (for 20 days), and 2) ozone; 51 patients were treated daily with ozone (generated by an OZOMED equipment, Cuba), 20 sessions, by rectal insufflation (with an ozone dose of 10 mg, ozone concentration: 50 mg/l) and locally. For local ozone treatment, the lesion was covered with a plastic bag, sealed to the leg, which was then put under vacuum, in order to eliminate the air inside it. Afterward, the bag was refilled with ozone at a concentration of 60 mg/l. The patient remained with the plastic bag for 1 h. After that, the bag was removed and the lesion was covered with ozonized sunflower oil (Oleozon®).

Debridement was indicated for essentially every wound and gauze dressings were used. Medical personnel were instructed to report all adverse experiences whether or not described in the package circulars of the study medications.

Blood samples for biochemical analysis were obtained after a 12-h overnight fast, at the beginning, and 24 h after the last

ozone and antibiotic treatments. Glucose, fructolysine, advanced oxidation protein products, nitric oxide, reduced glutathione, glutathione peroxidase, catalase, superoxide dismutase, total hydroperoxides, peroxidation potential, and malondialdehyde were measured.

The main variables considered were:

1) Clinical evaluation of the lesions: a) Measurement of the area and perimeter of the lesions by means of a trace done on an acetate plate (planimetric analysis), under aseptic conditions, at the beginning and at the end of the study, and the change in both parameters with time. The resultant area and perimeter were quantified using a computer program (DIGIPAT). b) Qualitative clinical evaluation of the lesions. c) Length of hospitalization was the time necessary to obtain an aseptic lesion, with good granulation tissue and in a healing process or ready to receive a graft.
2) Glucose levels, measured at the beginning and at the end of the study, taking into account that hyperglycemia is the primary factor, were associated with diabetes and its complications.
3) Secondary variables considered were: a) Serum levels of fructolysine, advanced oxidation protein products, nitric oxide, reduced glutathione, glutathione peroxidase, catalase, superoxide dismutase, total hydroperoxides, peroxidation potential and malondialdehyde. b) Side effects.

A good result was considered when there was a decrease in: the area and perimeter of the lesion, the duration of hospitalization, and in levels of glucose, fructolysine, advanced oxidation protein products, malondialdehyde, peroxidation potential, and total peroxides. An increase in nitric oxide, reduced glutathione, glutathione peroxidase and an approach to physiological values of the ratio catalase / superoxide dismutase were also considered as good results.

In the case of biochemical variables, laboratory data for healthy individuals ($n=50$) were taken as normal reference values (control group). This group of subjects corresponded in terms of age, sex and ethnicity with both groups of patients enrolled in the study.

Therapy was considered successful if 70% of the patients treated with ozone had a positive outcome, taking into account the main variables, and if this improvement was 20% higher than that in the patients treated with antibiotic therapy.

2.2. Biochemical determinations

All biochemical parameters were determined by spectrophotometric methods using an Ultrospect Plus Spectrophotometer from Pharmacia LKB. Glucose concentration was determined with a colorimetric method (the absorbance was measured at 560 nm), according to the procedure described by Schmidt and Pfeiffer (1989). Catalase activity was measured by following the decomposition of hydrogen peroxide at 240 nm at 10 s intervals for 1 min (Boehringer Mannheim, 1987). Superoxide dismutase and

glutathione peroxidase were measured using kits supplied by Randox Laboratories Ltd., Ireland (Cat. No. SD125 and No. RS505). Concentrations of malondialdehyde 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 586 nm. For standards, freshly prepared solutions of malondialdehyde bis [dimethyl acetal] (Sigma, St. Louis, MO, USA) were used and assayed under identical conditions (Esterbauer and Cheeseman, 1990). 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 (Bradford, 1976).

The peroxidation potential was measured by inducing lipid peroxidation by adding Cu^+ (2 mM) to serum (incubated for 24 h at 37 °C), in order to know the balance between prooxidant and antioxidant factors. The difference between malondialdehyde levels, measured at 0 and 24 h after induction, for each sample, was calculated (Özdermirler et al., 1995).

After precipitation of thiol proteins using trichloroacetic acid 10%, reduced glutathione was measured according to the method of Sedlak and Lindsay (1968) with Ellman's reagent [5′ 5 dithiobis (2-nitrobenzoic acid) 10^{-2} M (Sigma St. Louis, MO, USA)]; absorbance was measured at 412 nm. Nitrite/nitrate levels were determined by the Griess reaction after 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)-

Table 1
Baseline patient characteristics

Characteristics		Control ($n=49$)		Ozone ($n=51$)	
		n	%	n	%
Age	20–40	7	14	5	9
(years)	40–60	20	40	17	32
	≥60	22	44	30	57
Ethnicity	White	30	61	39	75
	Black	8	16	7	13
	Mixed race	11	22	6	11
Sex	Female	19	38	26	50
	Male	30	61	26	50
Previous	Hypertension [a]	23	46	20	38
history	Renal dysfunction [b]	2	4	2	3
	Cardiovascular disease [c]	7	14	10	19
ETD	$X\pm$S.D.	18±8		17±11	
(years)	Min.	1		1	
	Max.	42		50	

ETD is Evolution time of the disease, X is the mean value, S.D. is the standard deviation.
No significant statistical differences between both groups ($P>0.05$) for these studied variables were achieved.
  [a] Hypertension was defined as elevation of systolic (>140 mm Hg) and/or diastolic (>90 mm Hg) blood pressure.
  [b] Cardiovascular disease (CVD) was diagnosed by thorough history and physical examination.
  [c] Renal dysfunction: increase in serum creatinine >1.5 mg/dL.
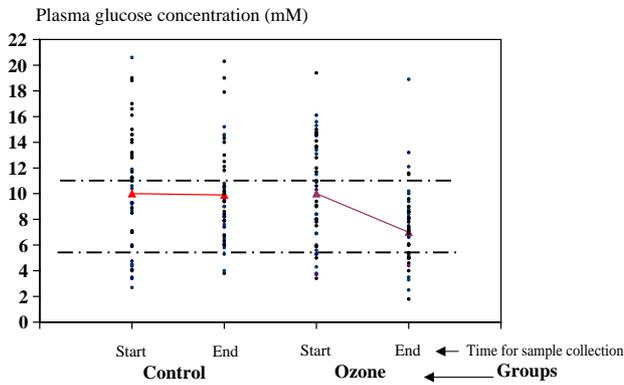
Plasma glucose concentration (mM)

Fig. 1. Plasma glucose concentrations (mM), at the beginning and at the end of the study (20 days), for control ($n=49$) and ozone ($n=51$) groups. The continuous lines correspond to the mean glucose concentrations for each treatment. The discontinuous lines (—) represent the normal reference interval for Cuban people (3.33–8.88 mM).

Table 2
Measurement of the area and perimeter of the lesions, at the beginning and at the end of the study, the variation in both parameters with time, as well as the expected total recovery for both groups

Parameter		Start ($X \pm$SD)	End ($X \pm$SD)
Area	Antibiotic ($n=49$)	54.84±0.39	40.72±0.35
(cm²)	Ozone ($n=51$)	57.97±0.52	23.31±0.36
	p^a	0.687	0.017
Perimeter	Antibiotic ($n=49$)	21.49±0.11	17.34±0.14
(cm)	Ozone ($n=51$)	18.49±0.14	12.62±0.13
	p^a	0.063	0.004
		Control ($n=49$)	Ozone ($n=51$)
Area	%	50.30±0,17	74.58±0.35
reduction	p^b	0.017	
(%)			
Perimeter	%	26.63±0.17	41.52±0.25
reduction	p^b	0.000	
(%)			
Healing	$X \pm$SD	1.21±0.01	2.66±0.05
rate with	p^b	0.005	
respect			
to area			
(cm²/days)			
Healing	$X \pm$SD	0.24±0.00	0.34±0.00
rate with	p^b	0.040	
respect			
to			
perimeter			
(cm/days)			
Expected	$X \pm$SD	45±11	21±10
total	p^b	0.002	
recovery			
(days) [a]			

Start and End are the beginning and end of treatment (after 20 days) with ozone or antibiotics. Data are mean±S.D.
p^a is the probability between groups, at the same time of treatment.
p^b is the probability between different treatments.
[a] Expected Total Recovery is a criterion of healing according to the planimetric evaluation. It represents the expected days needed to achieve total healing (trend to zero of the area and perimeter of the lesions).

ethylenediamine dihydrochloride in 0.25% phosphoric acid) was added (Granger et al., 1995). 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 were measured as the oxidation of iodide anion to diatomic iodine by advanced oxidation protein products (Witko-Sarsat et al., 1998). Relative fructolysine content (Amadori's product of glycated serum protein) was measured by using the redox indicator NBT at 530 nm (Thorme et al., 1996).

2.3. 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 a 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 statistical significance used was $P < 0.05$.

3. Results

3.1. General characteristics of the patients involved in the study

In relation to the baseline characteristics (Table 1), both groups were similar at randomization ($P > 0.05$). Forty-four percent of patients in both groups were older than 60 years and the majority was white. Their medical history was characterized mainly by hypertension. The disease developed between 1 and 50 years.

Concomitant treatments were those used to control hypertension (captopril in 31%, nifedipine in 10% and nitropental in 2% of patients, respectively), glycemica (glibenclamide 62% of patients), and cardiovascular disease (aspirin in 17% of patients). For glycemic control, 38% of the patients who did not receive glibenclamide were under dietary control. More patients were treated with hypoglycemic drugs in the group treated with ozone (80%) than in the group treated with

Table 3
Clinical evaluation and length of hospitalization for both groups of treatment

Parameters		Antibiotic ($n=49$)	Ozone ($n=51$)
Clinic	Cured	34/69	39/78
evaluation [a] (n/%)	Not cured	15/30	12/24
		N.S.	
Length	X	34	26
of hospitalization [b]	Min.–Max.	7–83	6–58
(days)	S.D.	18	13
		$^c p = 0.010$	

N.S.: no significant; S.D. Standard Deviation.
[a] Qualitative evaluation made by the physician.
[b] Time of hospitalization needed to achieve an aseptic lesion, with good granulation tissue, ready to receive a graft.
[c] McNemar test, a comparison between groups of treatment.

antibiotics (45%) ($P<0.05$). The choice of antibiotic used for therapy was determined by the result of the antibiogram and the creatinine value. The most common antibiotics were cephalosporins (83%), aminoglycosides, penicillin, and others such as sulphaprim and metronidazole.

3.2. Blood glucose concentration

At the beginning of the treatments, the mean blood glucose concentration, for all the patients enrolled in the study, was 10.11 ± 4.20 mM (min. 2.70; max. 20.60), with no significant statistical differences between the groups. At the end of the treatments (20 days), the group treated with ozone had a significantly lower blood glucose concentration ($P<0.05$) than the group treated with antibiotic therapy (Fig. 1). At the beginning of the study, the number of patients with glucose levels within the reference range (3.33–8.88 mM) was 15 (30%) and 14 (27%) in the antibiotic and ozone groups, respectively. However, at the end of the treatments (20 days) there were 13 patients (26%) and 29 (56%) in the control and ozone groups, respectively. At the beginning of the study, more patients in the ozone group (80%) than in the control group (45%) received hypoglycemic drugs. Nevertheless, their glucose levels were higher than the normal reference range, indicating resistance to hypoglycemic treatment. However, at the end of ozone treatment, glucose level had decreased to within the normal reference range (Fig. 1). Glucose levels were improved in 84% and 40% of the patients treated with ozone and antibiotics, respectively, with significant differences ($P<0.05$) in both groups.

3.3. Measurement of the area and perimeter of the lesion

The results obtained for these parameters are shown in Table 2. At the beginning of the study there was no significant differences between the two groups. At the end of the treatments, a decrease in the area and perimeter was achieved in both groups. However compared with the antibiotic group, the ozone group reached a significant decrease in the area and perimeter. In four patients of the antibiotic group, an increase in the area and perimeter of the lesion was noticed which did not occur in the patients treated with ozone.

The reduction in lesion area with time was significantly greater in the ozone group (2.66 ± 0.03 cm^2/days) than in the antibiotic group (1.21 ± 0.01 cm^2/days). The same trend was observed with the change in lesion perimeter with respect to time (Table 2).

As measure of improvement, the expected total recovery time, the time to obtain total healing (zero area and perimeter of the lesion), was calculated. This marker showed that patients treated with ozone achieved total recovery quicker than the patients treated with antibiotic (Table 2).

3.4. Clinical evaluation

No significant differences between both treatments were obtained in terms of the clinical evaluation, but there was an increase in the number of patients cured and a decrease in the number of non-cured patients in the ozone group, compared with the antibiotic group. The duration of hospitalization decreased in patients treated with ozone compared with the

Table 4
Biomarkers of oxidative damage to proteins, antioxidant–prooxidant balance and nitric oxide

Groups biomarkers		Negative control ($n=60$)	Antibiotic ($n=49$)	Ozone ($n=51$)
Fructolysine (relative	Time 0	50 ± 17 [a]	1393 ± 125 [b]	1354 ± 110 [b]
fructolysine content)	20 days	–	789 ± 60 [c]	250 ± 27 [d]
AOPP (μM of	Time 0	12.13 ± 0.93 [a]	19.08 ± 0.84 [b]	21.90 ± 0.84 [b]
chloramine)	20 days	–	19.66 ± 0.70 [b]	16.86 ± 0.36 [c]
GSH (μM)	Time 0	3.32 ± 0.62 [a]	2.84 ± 0.31 [b]	2.82 ± 0.29 [b]
	20 days	–	1.67 ± 0.15 [c]	2.80 ± 0.18 [b]
MDA (μM)	Time 0	1.80 ± 0.07 [a]	8.78 ± 0.85 [b]	9.04 ± 0.44 [b]
	20 days	–	10.12 ± 0.68 [b]	9.92 ± 0.94 [b]
PP (μM)	Time 0	7.63 ± 1.29 [a]	13.09 ± 1.51 [b]	14.62 ± 1.18 [b]
	20 days	–	14.36 ± 1.96 [b]	10.40 ± 1.73 [c]
Total hydroperoxide	Time 0	103.78 ± 17.71 [a]	139.12 ± 29.71 [b]	145.43 ± 31.61 [b]
(μM)	20 days	–	139.19 ± 25.23 [b]	106.30 ± 30.92 [c]
GPx (U/ml/min)	Time 0	30.28 ± 4.12 [a]	63.66 ± 7.25 [b]	66.35 ± 8.91 [b]
	20 days	–	59.22 ± 6.65 [b]	68.35 ± 8.38 [b]
SOD (U/ml/min)	Time 0	1.46 ± 0.14 [a]	0.97 ± 0.16 [b]	1.09 ± 0.13 [b]
	20 days	–	1.58 ± 0.16 [a]	6.87 ± 0.32 [c]
CAT (U/ml/min)	Time 0	161.5 ± 23.11 [a]	2880 ± 250 [b]	2112 ± 210 [c]
	20 days	–	2370 ± 247 [b]	3101 ± 290 [b]
Catalase/superoxide	Time 0	0.11 ± 0.20 [a]	2.24 ± 0.11 [b]	2.08 ± 0.10 [b]
dismutase	20 days	–	2.05 ± 0.11 [b]	0.51 ± 0.09 [c]
NO^{-3}/NO^{-2} (μM)	Time 0	67.82 ± 22.44 [a]	52.51 ± 9.78 [b]	50.06 ± 7.39 [b]
	20 days	–	75.31 ± 6.26 [a]	79.88 ± 6.26 [a]

AOPP, advanced oxidation protein products; GSH, reduced glutathione; MDA, Malondialdehyde; PP, peroxidation potential; GPx, glutathione peroxidase; SOD, superoxide dismutase; CAT, catalase; NO^{-2}/NO^{-3}, nitrites/nitrates. Data are mean±S.D. Means having different superscript letters indicate significant difference ($p<0.05$) between groups and within each group, comparing initial and final values.

Fig. 2. Events associated with diabetes, its complications and its relation with ozone therapy in the different stages of the pathological cascade. Legend: Pathways blocked by the use of ozone (X), pathways probably blocked by the use of ozone (?), increase (↑), decrease (↓), inhibition (−), activation (+). Ozone treatment improved glycemic control, prevented oxidative stress, standardized organic peroxide levels, and activated superoxide dismutase. The pharmacodynamic effect of ozone in the treatment of patients with neuroinfectious diabetic foot can be ascribed to the possibility of its being a superoxide scavenger.

and conversion of glucose by insulin-sensitive tissues (muscle and fat) are severely impaired. To overcome these defects, the release of insulin by β-cells is increased, resulting in typical type 2 diabetes, which means high glucose plasma level in the presence of hyperinsulinism (Rösen et al., 2001).

A direct link between the presence of oxidative stress and impaired glucose uptake has been demonstrated. In adipocytes, glucose uptake is rapidly decreased in the presence of hydrogen peroxide (H_2O_2), an effect was reversed by ozone treatment in preclinical (Al-Dalain et al., 2001) and clinical studies (Table 4). This reduction was accompanied by a decrease in phosphatidyl inositol 3 kinase activity and glucose transport 4 (GLUT4) translocation (Rudich and Tinosh, 1998).

Antioxidant depletion accompanied by a decrease in glucose uptake has been reported in type 2 diabetes patients. The findings of a 4-year prospective study led to the hypothesis that an imbalance in reactive oxygen species and antioxidants is an important pathogenic factor leading to insulin resistance, producing an impairment of the signaling pathway (Salonen and Nyyssonen, 1995). Additional clinical observations have shown a close association between oxidative stress and insulin sensitivity (Fesken and Vintanen, 1995; Ceriello et al., 2001).

If all these results and hypotheses are true, then we can postulate the inverse situation: an increase in antioxidant capacity should improve insulin resistance. This has been proven in several preclinical and clinical experiments in which α-lipoic acid caused an improvement in glucose utilization and insulin resistance (Jakob et al., 1996; Henrikson et al., 1997; Khamisi and Potashnik, 1997). It has been demonstrated in adipocytes and in muscle cells that (insulin receptor substrate I) IRS-I and phosphatidyl inositol 3 kinase are activated in the presence of α-lipoic acid. This produces a translocation of the GLUT4 transporter from the intracellular compartment to the cellular surface (Henrikson et al., 1997). It has been also demonstrated that α-lipoic acid treatment increases insulin-stimulated glucose oxidation and glycogen synthesis by 33% and 38%, respectively (Estrada and Ewart, 1996), combined with a significantly lower plasma concentration of insulin (15–17%) and free fatty acids (Khamisi and Potashnik, 1997).

We have shown that ozone treatment affected metabolic actions related to the inhibition of glycogen depletion (Candelario-Jalil et al., 2001) and a decrease in the availability of free glucose (Fig. 1). Furthermore, in ischemic situations, ozone administered by rectal insufflation inhibited ATP depletion ($P < 0.05$) (Peralta et al., 2000). It has been reported that the mitochondrial electron flux becomes uncoupled from ATP synthesis under hyperglycemic conditions, suggesting that normalization of the mitochondrial production of superoxide anion ($O_2 \cdot -$) blocks some routes of hyperglycemic damage (Nishikawa et al., 2000).

The preconditioning actions of ozone (León et al., 1998; Barber et al., 1999; Peralta et al., 1999, 2000; Candelario-Jalil et al., 2001), as well as the improvement in the antioxidant defense systems and the reduction in reactive oxygen species, favor an

appropriate redox balance, suggesting that the decreased hyperglycemia, observed in type 2 diabetes patients, could be related to an increase in insulin sensitivity and its signaling pathway. Nevertheless, other mechanisms could be present, since some inhibitors of aldose reductase have important antidiabetic effects. The interaction of 2,4-tiazolidinediones by-products with the family of activated receptors results in peroxisomal proliferation (Constantino et al., 1997) or in insulin secretion in the case of IAR M-16209 agent (Wrobel et al., 1992).

The severe vascular damage occurring in a neuroinfectious diabetes foot, which is associated with hyperglycemia, promotes ulcers and infections, often leading to the need for amputation. For this reason, the control of this series of events is very important. Both treatments (antibiotic and ozone) favored cicatrization. However, ozone treatment exerted a more powerful effect. The superiority of ozone treatment (Figs. 1 and 2, Tables 2–4) is a direct consequence of its "antidiabetic effects" (already analyzed, and are not present in antibiotic therapy), its capacity to maintain the cellular redox balance (absent with antibiotic therapy as a direct effect) and its antimicrobial property, which is common to both treatments and the main reason for the use of antibiotic therapy.

It is important to consider that among the "non-cured" patients, 7 (16%) were treated with antibiotics under amputation compared with 3 (5%) which were treated with ozone (Table 3). Ozone therapy not only reduced the number of patients needing an amputation, but also decreased the extent of the amputation. Amputation has a deep social significance in relation to the patient's quality of life and thus any therapy that reduces the need for it and its extent has great advantages.

The fructolysine content of tissues, due to Maillard reactions, is associated with oxidative processes. Fructolysine is a precursor of the advanced oxidation protein products, which are induced by oxidative stress, and also induces oxidative stress (Kasper and Frenk, 2001). Glycolated proteins inactivate enzymes (for example, the antioxidant enzymes) and also affect the functions of bonding, transportation and protein structure (García Villanova, 1994). Both treatments decreased the fructolysine content by the end of treatment, without achieving reference levels but the content in the ozone group was significantly different from that in the antibiotic group and close to the content in the control group. The aim of antibiotic treatment is to reduce and eliminate the infectious process present in the diabetic foot. It is well known that during infection there are reactive oxygen species, which are also the result of inflammation, associated with activated neutrophils and macrophages (Mushova et al., 1999; Mazade and Eduards, 2001). These conditions favor the Maillard reactions. The decrease in the fructolysine content in the patients treated with antibiotics suggests that this result is a consequence of the reduction or elimination of the infection, decreasing the reactive oxygen species capable of generating Amadori compounds.

The results for the group treated with ozone indicate that its superior efficacy compared to antibiotics is due not only to its antimicrobial effect, but also to its demonstrated capacity to reduce hyperglycemia. Other factors that influence this efficacy are related to its capacity to up-regulate the polyol pathway and the formation of fructose, which interacts with NH_2 groups of proteins to form fructolysine (Al-Dalain et al., 2001).

H_2O_2 is a reactive oxygen species produced during glucose autoxidation (Elgawish et al., 1996). The capacity of ozone to restore the concentrations of organic peroxides to normal levels was different from that of antibiotics and is of significance to its "antidiabetic" effects (Table 4). The H_2O_2 derived from hyperglycemia has been demonstrated to promote cell death by necrosis in human aortic smooth muscle cells, and this effect was reversed only when catalase was added to the culture medium (Peiro and La Fuente, 2001). H_2O_2 is capable of activating the transcription factor nuclear factor κB (NF-κB), which promotes the generation of cell adhesion molecules, cytokines and pro-coagulant tissue factor, mediators of the vascular complications present in diabetic patients. The participation of oxidative stress in the activation of NF-κB was well demonstrated when this factor was inhibited by antioxidants such as vitamin E and α-lipoic acid (Bierhaus et al., 1997; Rösen et al., 2001).

Thus we have found that ozone treatment, by means of its oxidative preconditioning effect, normalizes glucose levels and consequently restores the concentrations of organic peroxides. It also contributes to the control of the vascular complications of neuroinfectious diabetic foot, by the above mechanisms and its likely actions on transcription factor NF-κB.

Neither treatment restored the glutathione concentrations to those of the control group. Reduced glutathione levels reached the initial concentration at the end of ozone treatment, but the advanced oxidation protein products had decreased significantly. However, in the group treated with antibiotics an additional depletion of reduced glutathione was observed, with no modification in the advanced oxidation protein products. The decrease in reduced glutathione levels in this group is related to the changes of glutathione peroxidase in the ozone group, although at the beginning of the study glutathione peroxidase concentrations were higher than in the reference group.

As an expression of the antioxidant–oxidant balance, the peroxidation potential was positively affected in the group treated with ozone compared to the antibiotic group (it maintained its same level at the end of the treatment). A significant decrease compared to the initial level was found in the ozone group but levels were still significantly different from those in control group.

It is well known that superoxide dismutase and catalase are scavenger enzymes of reactive oxygen species and that they can be inhibited during oxidative stress. Serum total antioxidant activity, as an expression of the balance between the generation and inactivation of these oxidized metabolites, has been demonstrated to be decreased in diabetic patients (Ceriello et al., 2001). Our results and the literature indicate that there must be a relation between superoxide dismutase

and catalase levels that guarantees an effective control of oxidative stress. The ratio catalase / superoxide dismutase is considered as a biomarker of glycemic control and as a risk factor in the development of diabetic complications (Sozmen et al., 2001). While after therapy with antibiotics the ratio catalase / superoxide dismutase was not modified, with higher levels being measured at the end of the study, after ozone treatment the ratio was decreased by 24% compared to its initial value. This ratio has been shown to be proportional to the increase in glycosylated hemoglobin as a biomarker of long-term glycemic control. The results obtained in the ozone group for the ratio catalase / superoxide dismutase indicated an improvement in glycemic control and was related to the fructolysine content because the concentrations of the Amadori products of glycated serum protein represent the mean plasma glucose concentration of the last 2–3 weeks (García Villanova, 1994).

Superoxide dismutase is a scavenger of $O_2\cdot^-$, transforming it in H_2O_2, and is well regulated by ozone treatment. The overproduction of $O_2\cdot^-$ has been recently proposed as an important factor that unifies the four hypothesis about the physiopathologic mechanisms responsible for diabetic complications (Du, 2000). Not only in this study but also at an experimental level (Al-Dalain et al., 2001) was a significant increase in superoxide dismutase, even higher than that in the control group, seen in the ozone group by the end of treatment. These results indicate that the overproduction of $O_2\cdot^-$ by the mitochondrial electron-transport chain must be reduced by ozone treatment, decreasing the concentration of advanced glycation end-products, the activation of PKC, the increase in hexosamine pathway flux and the activation of the polyol pathway.

The regulation of aldose reductase achieved with ozone treatment can be the result of a decrease in glucose concentrations, as observed in preclinical studies (Al-Dalain et al., 2001) and from our results because of the non-accumulation of glycolytic metabolites and the disappearance of GAPDH inhibition by superoxide. The hyperglycemia-induced overproduction of mitochondrial $O_2\cdot^-$ induces a 66% decrease in GAPDH activity (Du, 2000).

The increase in superoxide dismutase activity obtained with ozone treatment could be the result of a stimulation of the gene expression of the gene encoding this enzyme. The effect of ozone on the novo synthesis of proteins has been demonstrated experimentally (Rahman et al., 1991; Punjabi et al., 1994; Cardile et al., 1995).

The NO^{-2}/NO^{-3} ratios, a measure of nitric oxide, returned to normal values by the end of the study with both treatments. This result suggests that both treatments control the infection process and that antibiotic, unlike ozone, do not substantially modify the cellular redox state or hyperglycemia. An integrated diagram of the events associated with diabetes and its complications and its relation to ozone therapy is shown in Fig. 2. Some of the pathways were blocked by the use of ozone treatment with a positive result at the end of the study. Clinically, patients treated with ozone therapy had a better and faster recovery of their lesions compared to the patients treated

with antibiotic therapy (26 days vs. 34 days). No side effects were found.

A preliminary economic evaluation showed that the use of ozone therapy in the treatment of neuroinfectious diabetic foot produced a decrease in treatment costs of about 25% compared to the use of antibiotics. Taking into account that the probability of hospitalization and the cost to health care is 3 to 5 times higher for diabetic patients than for non-diabetic patients and that epidemiological studies suggest that there will be 300 million diabetic patients in the year 2010 (Bjork, 2001), further innovations in diabetes therapy are needed to improve treatment and its cost. Ozone therapy could provide a solution to diabetes therapy.

In summary, ozone treatment of patients with diabetes type 2 suffering from neuroinfectious diabetic foot improved glycemic control by decreasing hyperglycemia, increasing insulin sensitivity and preventing oxidative stress associated with diabetes mellitus and its complications. Ozone therapy could be a future alternative in the therapy of diabetes and its complications.

Acknowledgments

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