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Original Article

Rectal insufflation of ozone attenuates chronic oxidative stress in elderly patients with cardiovascular diseases

Irina Wilkins-Perez[1], Livan Delgado-Roche[2], Juan M. Barrios[1], Gloria B. Fabregas-Popoco[1]

[1]Laboratory of Oxidative Stress, Mexican Association of Oxidative Stress (AMEOX), Mexico City, Mexico, America, [2]Department of Pharmacology, Center of Marine Bioproducts (CEBIMAR), Havana 10600, Cuba, America

Address for correspondence: Livan Delgado-Roche, Loma St. and 37, Nuevo Vedado, Plaza de la Revolucion, Havana 10600, Cuba, America. E-mail. ldelgadoroche@gmail.com

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ABSTRACT

Objective: Aging is a biological inevitable process, characterized by a progressive deterioration of physiological functions and metabolism, ultimately leading to death, meanwhile cardiovascular diseases (CVD) are the most common cause of death in aged subjects. Chronic oxidative stress contributes to age-associated cardiovascular dysfunctions and diseases. Because ozone therapy can modulate the antioxidant system, the aim of this study was to investigate the therapeutic efficacy of ozone in aged patients with CVD. **Materials and Methods:** A randomized controlled clinical trial was performed with 30 patients older than 60 years with a diagnosis of any cardiovascular disease. A parallel group ($n = 40$) age and gender matched was used as a reference for the determined variables. Patients were treated with 200 ml ozone/oxygen mixture at 20 μg/mL of ozone by rectal insufflation once a day during 15 days. **Results:** Ozone improved the antioxidant status of patients by reducing biomarkers of protein and lipid oxidation and regulating the oxidant/prooxidant balance. **Conclusion:** Medical ozone treatment could be used to ameliorate the redox disruption in aged patients and as a complementary therapy in the treatment of CVD and its complications.

KEY WORDS: Aging, cardiovascular diseases, medical ozone, rectal insufflation

INTRODUCTION

Aging is a biological inevitable process, characterized by a progressive deterioration of physiological functions and metabolism, ultimately leading to death [1]. Cardiovascular diseases (CVD) represent the most prevalent chronic diseases in aged subjects. The World Health Organization reported that CVD represent the first cause of death worldwide [2].

The free radical theory of aging proposes that free radicals, a by-product of normal metabolism, cause oxidative damage to macromolecules [3]. Their over-production causes cellular dysfunction with age and eventually cell death. Indeed, it has been shown by several studies that an increase of oxidation products, like oxidized proteins and lipids, correlates with age [4-6].

Historically, scientists have been trying to manipulate the lifespan of organisms by modulating reactive oxygen species (ROS) metabolism, mainly at the level of scavenging systems, through exogenous and/or endogenous interventions. The supplementation with antioxidants, caloric restriction, and physical activity have been used to test the free radical theory of aging; and finally, to reduce the impact of age-related dysfunctions [7]. In a recent review, Bocci *et al.* [8] pointed out that any change of the environment perturbs the body homeostasis but, whether the stress is tolerable, the body can adapt to it and may survive or improves its functions. Evidences that antioxidant enzymes, nitric oxide pathways, and other subcellular activities could be modulated by low ozone doses is now proven and could support the surprising effects of ozone in many pathological conditions [9-15].

The mechanism of action of ozone is closely linked to the production of reactive molecules, by reacting with the membrane phospholipids: Ozonides, aldehydes, peroxides and hydrogen peroxide (H_2O_2). These molecules react with substances' double bonds present in cells, fluids or tissues. Furthermore, reactive molecules interact with DNA and cysteine residues of proteins [16], acting as second messengers, able to activate antioxidant enzymes, chemical and immune-response mediators and cytokines [17]. On the other hand, ozone can activate nuclear transcription factors, such as the nuclear factor erythroid 2 (Nrf2), which in turn induces antioxidant response

elements [18]. Recently, Re *et al.* [19] demonstrated the *in vivo* activation of the Nrf2 pathway by a low dose of ozone and the promotion of the feedback mechanism that induces the synthesis of proteins which collectively favors cell survival. Thus, the aim of this study was to evaluate the efficacy of ozone therapy in reducing the oxidative stress index in elderly subjects with CVD.

MATERIALS AND METHODS

Study Design

This clinical trial was carried out in accordance with the principle of the Declaration of Helsinki [20]. 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 scheme of treatment.

Adult patients of both gender and different ethnic origin older than 60 years, with CVD who were attended in the Cuban Medical Services (Mexico City, Mexico) were eligible to participate in the study. Exclusion criteria were: Diabetes mellitus, severe septic conditions, hypersensitivity to the medication to be used, hepatic dysfunction, renal failure (serum creatinine level in male >1.4 mg/dl, in female >1.1 mg/dl), 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. As control group, 40 age-matched healthy subjects (40-65 years old) were used to establish the normal reference interval of oxidative stress biomarkers.

Ozone was generated by Ozomed Plus equipment (National Center for Scientific Research, CNIC, Havana, Cuba), and was administered by rectal insufflation. Ozone was obtained from medical grade oxygen, and was used immediately upon generation and represented only about 3% of the gas mixture (O_3/O_2). The ozone concentration was controlled in real time by using a built-in UV spectrophotometer at 254 nm, as recommended by the Standardization Committee of the International Ozone Association.

Patients ($n = 30$) were treated with 200 ml of O_3/O_2 containing 20 μg/ml of ozone once a day during 15 days. Nelaton catheter (Visa Laboratory S.A. de C.V., Mexico) was introduced 10-15 cm by rectal way to deliver the gas for 5 min. The patients were encouraged to empty his or her bladder and bowels before the procedure.

Blood samples for biochemical analysis were obtained after a 12 h overnight fast, at the beginning and 24 h after the last dose of medical ozone. The samples were immediately centrifuged at 3000 g, at 4°C for 10 min. The serum was collected and aliquots were stored at −70°C until analysis.

Biochemical Determinations

All biochemical parameters were determined by spectrophotometric methods using a Spectrophotometer Gensys 6 (Thermo Scientific, USA). Superoxide dismutase (SOD) activity was measured using kits supplied by Randox Laboratories Ltd. (Ireland; Cat. No. SD125 and No.RS505). Glutathione peroxidase (GPx) activity was measured as previously described [21]. After precipitation of thiol proteins using trichloroacetic acid 10%, reduced glutathione (GSH) was measured using the Ellman's reagent (5,5-dithiobis [2-nitrobenzoic acid]) (Sigma St. Louis, MO, USA) at 412 nm [22]. Concentrations of malondialdehyde (MDA) were measured at 586 nm using the lipid peroxidation (LPO)-586 kit obtained from Calbiochem (La Jolla, CA, USA). The levels of proteins' carbonyls groups (CG) were measured as previously described [23]. CG present in the samples react with 2,4-dinitrophenyl hydrazine forming a hydrazine derivate detectable at 375 nm. Total serum cholesterol was determined using a commercial kit (Spinreact S.A./S.A.U.; Gijona, Spain).

Statistical Analysis

Data were analyzed using the SPSS software version 18.0 (SPSS Inc, Chicago, IL, USA). One-way ANOVA, followed by Bonferroni test was employed to determine differences between groups. Results are the means ± standard deviation. The level of statistical significance was set as $P < 0.05$.

RESULTS

A high prevalence of risk factors for CVD, such as hypertension (64%), hypercholesterolemia (53%), obesity (33%) and smoking (27%) was noted in aged patients. Meanwhile, cardiovascular disorders such as ischemic cardiopathy (67%) and myocardial stroke (3%) were present. The full characterization of studied population is shown in Table 1.

Before ozone treatment, plasmatic oxidative stress parameters were determined in order to characterize the redox status in aged patients. The results showed a significant diminishing ($P < 0.05$) of SOD and GPx activity, as well as lower levels of GSH in comparison with healthy subjects. In accordance with a disruption of antioxidant mechanisms, a significant increase ($P < 0.05$) of oxidative damage to lipids (MDA) and proteins (CG) was detected, indicating the presence of a pathologic oxidative stress. After the rectal insufflation of ozone, MDA and CG levels were significantly reduced ($P < 0.05$), showing no statistical differences compared with the control group. At the same time, ozone was able to restore the antioxidant defenses by improving the antioxidant status of these patients [Table 2]. These results demonstrated that chronic oxidative stress, associated with cardiovascular disorders, could be regulated with ozone therapy in aged patients.

Table 1: Baseline characterization of subjects

Characteristics	*n* (%)	
	Control group (*n*=40)	Ozone group (*n*=30)
Age (years)		
40-65	40 (100)	3 (10)
66-75	0 (0)	26 (87)
75-80	0 (0)	1 (3)
Gender		
Female	26 (65)	19 (63)
Male	14 (35)	11 (37)
Previous History		
Myocardial stroke	0 (0)	1 (3)
Ischemic cardiopathy	0 (0)	20 (67)
Risk factors		
Hypertension[a]	0 (0)	19 (64)
Hypercholesterolemia[b]	0 (0)	16 (53)
Obesity[c]	0 (0)	10 (33)
Smoking	0 (0)	8 (27)
Complementary diagnosis		
TC (mg/dl)	176.25±1.31	269.38±2.94
BMI (kg/m^2)	24.73±1.85	32.45±3.12

Table shows the baseline characteristics of both groups involved in the study: (a) hypertension was defined as elevation of systolic (>140 mmHg) and/or diastolic (>90 mmHg) blood pressure; (b) hypercholesterolemia was defined as increase in total serum cholesterol (>239 mg/dl); (c) subjects with BMI values higher than 27 kg/m2 were considered obese, TC: total cholesterol, BMI: Body mass index

Table 2: Effect of ozone rectal insufflation on plasmatic biomarkers of oxidative stress

Biomarker	Control group (*n*=40)	Before ozone therapy (*n*=30)	After ozone therapy (*n*=30)
SOD (IU/l)	384±12.5[a]	248.43±17.59[b]	1406.71±109.55[c]
GPx (IU/l)	460±23.21[a]	25.1±7.51[b]	501.29±36.88[a]
GSH (μmol/l)	9.93±2.51[a]	4.34±1.09[b]	10.65±0.92[c]
MDA (μmol/l)	0.82±0.2[a]	2.23±0.22[b]	0.71±0.11[a]
CG (nmol/l)	1.01±0.01[a]	1.6±0.16[b]	0.97±0.09[a]

Values are means±SD of oxidative stress biomarkers. Ozone treatment improved the antioxidant status by reducing the oxidative damage to lipids and proteins. Different letters represent statistical differences (ANOVA and post hoc Bonferroni, $P<0.05$). SOD: Superoxide dismutase; GPx: Glutathione peroxidase; GSH: Reduced glutathione; MDA: Malondialdehyde; TH: Total hydroperoxides; CG: Carbonyl groups of proteins, SD: Standard deviation, [a,b,c]statistical differences

DISCUSSION

Aging is a multifactorial process modulated by the interplay between genetic and environmental factors [24]. It is characterized by a physiological deterioration with time, reduced ability to respond adaptively to environmental stimuli, impaired homeostasis, and increased vulnerability to diseases, increasing mortality [25]. Age-related accumulation of cellular damage and death has been linked to oxidative stress, which promotes protein, lipid and DNA oxidation [26]. The strategies aimed to reduce age-related oxidative stress may improve the quality of life in older adults. In this scene, medical ozone represents a plausible therapeutic complement to reducing the oxidative stress associated with organism deterioration during aging.

Scientific evidences have demonstrated the efficacy of ozone therapy and its pharmacological actions in many human diseases. Oxygen metabolism, oxidative stress, autacoids release, general metabolism, the immune response and bactericide actions are improved by medical ozone [27]. In the present work, we used a low dose of ozone by rectal insufflation. Indeed, this administration route is increasingly being used as a systemic therapeutic protocol, viewed as an alternative to major auto-hemotherapy [28]. In addition, the biological effects of the rectal insufflation of ozone have been demonstrated extensively either experimentally or clinically [29-34].

Ozone acts in a hormetic mechanism and as a mild oxidant stressor, inducing the formation of second messengers, such as hydrogen peroxide and lipoperoxide compounds [35,36]. Low levels of LPO end-products induce cellular adaptive responses, inducing tolerance against subsequent oxidative stress by up-regulation of antioxidant mechanisms. LPO-end-products as well as ROS have been shown to play a key role as a regulator of genes expression [37]. In this scenario, ozone-derived reactive molecules are able to activate transcription nuclear factors, including Nrf2 which interacts with the antioxidant response elements, leading to the synthesis of a great variety of antioxidant enzymes able to restore the redox homeostasis, including GPx [38].

In accordance with the above cited reports, in the present work we showed new evidences on the regulatory effects of ozone therapy on the antioxidant systems. The increase in SOD and GPx activity promoted by ozone insufflation could be the result of a stimulation of the expression of genes encoding these enzymes. This effect could be associated with ozone's action on the novo synthesis of proteins, which has been demonstrated experimentally [39], and, in addition, with an activation of Nrf2 [19]. A recent study of our group demonstrated that ozone therapy induces the GPx1 gene expression, at the same time that promotes a preservation of GSH in atherosclerotic apolipoprotein E (ApoE) deficient mice [40]. The preservation of GSH is critical for vascular protection in patients with cardiovascular disorders [41]. Ozone treatment promoted an increase of GSH levels, which might contribute to a redox regulation impeding the oxidative modification of low-density lipoproteins (LDL). The oxidized LDL promotes a pro-inflammatory response leading to endothelial dysfunction and the progression of cardiovascular disorders [42]. A recent experimental study showed that the anti-atherosclerotic effect of ozone was associated with regulation of vascular oxidative stress [15]. Furthermore, clinical studies demonstrated that ozone therapy reduced the LPO of LDL isolated from patients with coronary artery disease, improving cardiovascular functions [43].

The improvement of antioxidant status by ozone therapy was accompanied by a reduction of proteins and lipid oxidation. One of the most often used technique to determine the oxidative damage on lipids is the spectrophotometric detection of MDA [44]. Many studies revealed a significant age-related increase in the plasma concentration of MDA [45,46]. On the other hand, CG is a generic marker of protein oxidation that are accumulated during the aging process. The age-related accumulation of oxidized proteins may reflect age-related

increases in rates of ROS generation, decreases in antioxidant activities or losses in the capacity to degrade oxidized proteins [47]. In the current study, MDA and CG were decreased after ozone insufflation, indicating the protective effect of medical ozone in patients with cardiovascular disorders. This effect could attenuate endothelial dysfunction, preventing the occurrence of adverse cardiovascular complications.

In summary, the results of this study demonstrated that ozone therapy contributes to reduce the chronic oxidative stress in patients with cardiovascular disorders through an improvement of the antioxidant mechanisms and a reduction of oxidized macromolecules. The present study reinforces the efficacy criteria of medical ozone as a therapeutic complement to prevent the progression and reduce the complications of CVD.

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