Original Article

Effect of Ozone Therapy on Antioxidant Response in Patients with Diabetic Peripheral Neuropathy

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Abstract

Background: Type 2 diabetes mellitus is a chronic degenerative disease with multiple complications, including peripheral neuropathy. One of the biochemical mechanisms that explain its pathogenesis and development is oxidative stress. This study aimed to evaluate the oxidative status in diabetic patients with peripheral neuropathy before and after treatment with ozone. **Methods:** A total of 25 patients with diabetic peripheral neuropathy were recruited in this study. The disease was classified with the modified Neuropathy Michigan Scorë instrument. Ozone was administered in these patients (27ug/ml) in 12 sessions and the binding activity of Nrf2/ARE, oxidative damage and antioxidant capacity was assessed in their plasma. **Results:** The feet and thighs were found to be the most affected sites (92%), with predominating symptoms such as burning, pain, stitches, numbness and cramps. After ozone therapy, a decrease in these symptoms, HbA1c and binding activity of Nrf2/ARE ($p<0.05$) was observed. However, the antioxidant capacity and oxidative damage were found to be maintained. **Conclusions:** Ozone treatment in patients with diabetic peripheral neuropathy decreased the severity of the symptoms.

Keywords: Diabetes Mellitus, Neuropathy, Ozone Treatment, Oxidative Stress

1. Introduction

Type 2 diabetes mellitus (DM) is a metabolic disorder of multiple etiologies characterized by chronic hyperglycemia. Additionally, alterations in the metabolism of carbohydrates, fats and proteins occur in DM patients due to defects in insulin secretion, insulin action, or both.[1] In 2012, the number of people with diabetes was 62 million in America (422 million people worldwide), and according to the Pan American Health Organization, this number will reach 109 million in 2040. In 2019, DM was the sixth leading cause of death with an estimated 244,084 deaths and the second leading cause of Disability Adjusted Life Years (DALYs).[2] In Mexico, 8.6 million of the adult population suffered from DM in 2018. The prevalence of this disease is expected to continue increasing as the population ages and it could be higher because a high proportion of the population is also overweight or obese.[3] The disease was found responsible for 151,214 deaths in Mexico in 2020.[4]

DM is a complex disease that consists of metabolic alterations of multiple etiologies characterized by chronic

Access this article online

Quick Response Code:

Website: www.jnsbm.org

DOI: https://doi.org/10.4103/jnsbm.JNSBM_14_1_1

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Submitted: 28th August, 2022 **Received:** 30th August, 2022
Accepted: 22nd September, 2022 **Published:** 25th January, 2023

How to cite this article: Guadalupe V R A, Humberto M V R, Saul A B A, Enrique V R L, Graciela Z G, Laura B L, Angelica Q E M. Effect of Ozone Therapy on Antioxidant Response in Patients with Diabetic Peripheral Neuropathy. J Nat Sc Biol Med 2023;14:1-9

hyperglycemia. It is a multisystemic organic syndrome with different phenotypic characteristics, genetic predisposition and defects in secretion and action of insulin. Multiple factors effect the etiopathogenesis and evolution of this disease such as advanced age, excessive calorie consumption, overweight, presence of central adiposity and sedentary life. The disease is evolved slowly with unclear clinical investigation in the initial stage. However, later it evolves into notable chronic complications at the micro and macrovascular levels, such as retinopathies, nephropathies, cardiovascular diseases and many other neuropathies.[5-8]

Diabetic peripheral neuropathy (DNP) is a highly prevalent condition that substantially affects patients by causing pain and reducing the quality of life.[9] Some studies suggest that the incidence of DNP increases with the duration of diabetes and is related to poor glycemic control, hyperglycemic-oxidative stress and the production of inflammatory cytokines.[10-12] At least 50% of all diabetic patients develop neuropathy, which is also the most common cause of foot ulcers and non-traumatic amputations in the western world.[13] DNP is classified into several syndromes, each with a distinctive pattern of peripheral nerve involvement. At the time of diagnosis, these syndromes can be found either as coexisting or they can also occur one after another in the same patient. Painful DDN can manifest as pain with nuances like burning, cutting, abnormal electrical sensations (cramps), with sensation of cold, compression or hyperalgesia predominantly at night.

An accurate diagnosis of DNP is essential to prevent the development of clinical manifestations. The Michigan Neuropathy Screening Instrument (MNSI) is the most widely used diagnostic tool because it is simple, validated and strongly correlated with electrophysiological parameters. This instrument consists of a questionnaire and clinical examination with a score of 8 points including inspection, vibratory sensitivity and achilles reflexes studies. A score greater than 2 points is considered as positive neuropathy.[14]

The Clinical Practice Guidelines (GPC) in Mexico recommends primary management in adults who are presented with pain due to DNP.it's The initial management includes glycemic control, administration of analgesics, non-steroidal anti-inflammatory drugs and tricyclic antidepressants. Moreover, it includes selective serotonin reuptake inhibitors such as duloxetine and venlafaxine and antiepileptics having central and peripheral action. These antiepileptics decrease nerve excitability such as carbamazepine, gabapentin and pregabalin.

Several molecular mechanisms have been proposed to explain how hyperglycemia and the metabolic state of DM can cause damage and alterations in the oxidative state. It has been described that oxidative stress is essential for proper redox signaling, genome regulation, cell growth and division. Its alteration leads to the modification of the oxidative state of the cell.[15] Increased oxidative stress is a widely accepted feature in the development and progression of diabetes and its complications, such as NPD, as it is often accompanied by increased free radical production or impaired antioxidant defenses.[16-18] The increased production of free radicals in diabetes may be detrimental through several mechanisms which have not been fully explored. These include direct damage to blood vessels leading to nerve ischemia and facilitation of reactions of advanced-glycation end-products (AGEs).[13] Nuclear-related factor 2 (Nrf2) or nuclear-related erythroid-derived 2 has been described as a connector of these metabolic pathways.[19] It acts as a transcription factor promoting the activation of cytoprotective enzymes. Moreover, it regulates the inducible expression of several genes encoding detoxifying and antioxidant enzymes by binding to a specific DNA sequence known as Antioxidant Response Element (ARE).[20] However, due to the low levels of this protein in patients with DNP, there is a decrease in ARE activity promoting the signaling of neuroinflammatory pathways.[21] Using natural and synthetic activators for Nrf2 could have therapeutic importance in DNP management. Most of these activators interact with Keap1 cysteine thiol residue facilitating the release of Nrf2 from the Keap1/ Nrf2 protein complex.[21] However, there are currently no specific medications available for DNP; therefore, it is crucial to investigate new therapeutic strategies for this disorder.

Ozone (O_3) therapy is a noninvasive and non-pharmacological procedure that has no side effects and is based on the regenerative capacity of O_3 for the treatment of various diseases. Different studies have shown the effectiveness of this therapy as a treatment for cardiovascular, peripheral vascular, neurological degenerative, orthopedic, gastrointestinal, genitourinary pathologies and multiple sclerosis. In addition, fibromyalgia, skin diseases, wound healing, diabetic ulcers, infectious diseases, pulmonary diseases and osteomyelitis have also beed treated with O_3 therapy.[22-31] Moreover, preclinical, genotoxic, toxicological and clinical studies support the application and safety of this medical therapy in a wide range of doses. Since the 20th century, O_3 therapy has been accepted by doctors as a non-traditional treatment due to its safety, convenience and low cost.[32]

The mechanism of action of O_3 is closely related to the production of ROS i.e. reactive oxygen species. The ROS formation in plasma is swift (milliseconds) accompanied by a transient and slight decrease in the antioxidant capacity depending on O_3 concentration. This antioxidant capacity returns to normal after 15-20 minutes, at which point hydrogen peroxide and other mediators diffuse into the erythrocytes, leukocytes and platelets, thereby; activating different metabolic pathways. Hydrogen peroxide and other reactive species act as signaling molecules in the intracellular medium, and therapeutic doses of O_3 generates controlled oxidative aggression that exacerbates a defense reaction. Studies have reported an increase in the activation of the transcriptional factor mediating Nrf2 through moderate oxidative stress caused by O_3.[33-35]

With this antioxidant capacity of O_3 therapy in different diseases, this study aimed to evaluate the antioxidant effect of ozone therapy in patients with DNP.

2. Materials and Methods

This study included 25 Mexican mestizo participants of both sexes from the Mexican Institute of Social Security (IMSS) and the Pain and Herniated Disc Clinic Dr. Milla Villeda's in Durango, Dgo, Mexico. The patients who visited this institute and clinic from May to November 2018 were enrolled into the study. The patients with DM and DNP were diagnosed with Neuropathy Michigan Score which is an internationally accepted measurement instrument. Patients who were taking antioxidant supplementation, pregnant or lactating women and chronic alcohol users were excluded from the study. After explaining purpose of the study to the patients, a written informed consent was obtained from them.

2.1 Blood sample collection

The study population was characterized by DM's and DNP's sociodemographic, prevalence, and evolution time. The blood and urine samples of the patients were taken during fasting condition and thereafter the samples were clinically analyzed Blood samples were collected in heparinized tubes and were centrifuged at 3500 RPM for 10 minutes and plasma and erythrocytes were separated for different tests. Plasma was used to determine glycated hemoglobin (HbA1c), glucose, urea, creatinine, urea nitrogen, albumin, cholesterol and lipid profile by dry chemistry. The oxidative damage in lipids, antioxidant capacity and Nrf2/ARE determined later.

Ozone was administered in the participants through rectal insufflation and each participant received 12 sessions. A mixture of oxygen and ozone in a dose between 25-35ug/ml was used for approximately 35 days i.e. applied every third day. At the end of the ozone treatment, the MNS instrument was applied again and biochemical tests were performed.

2.2 Lipid peroxidation measurement

Lipid oxidation in plasma was measured by the concentration of thiobarbituric acid-reactive substances (TBARS). Plasma was resuspended in phosphate buffer which was composed of 4.3mM Na2HPO4, 137mM NaCl, 2.7mM KCl, 1.4mM KH2PO4, 0.01M butylhydroxytoluene (BHT) and 30% trichloroacetic acid. The pH of the buffer was maintained at 7.4 After resuspension, the mixture was incubated at 4°C for 2 hours, after which it was centrifuged at 2000RPM for 15 min. The supernatant containing malondialdehyde (MDA) was collected and 0.1mM EDTA and 1% TBA was added into it. The mixture was incubated at a boiling temperature in a water bath for 15 min. The absorbance of the mixture was determined at 532nm in a UV/VIS spectrophotometer [model DU 650; Beckman].[36]

2.3 Total Antioxidant Capacity in plasma

Total antioxidant capacity was determined in plasma by colorimetric assay using 2,20-Azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS). The assay is based on the ability of the antioxidants present in the sample to inhibit the ABTS oxidation to ABTS*+ by a peroxidase.The amount of ABTS*+ produced was evaluated by reading the absorbance at λ 450 nm in a UV / VIS spectrophotometer [DU 650; Beckman]. The absorbance is inversely proportional to the antioxidant concentration. The antioxidant capacity in the plasma to prevent ABTS oxidation was compared to Trolox (water-soluble tocopherol analog).[37]

2.4 Nrf2 Binding Activity Assay

The DNA was extracted using the CTAB/DTAB DNA extraction method as described by Gustincinch in 1991 and Nrf2/ARE activity was determined using a commercial kit (TransAm® Nrf2, Active-Motif., Carlsbad, CA, USA). The kit contained immobilized oligonucleotide (5'-GTCACAGTGACTCAGCAGAATCTG-3) in the wells. The active form of Nrf2 present in the nuclear extract bound specifically to this oligonucleotide. The primary antibody used to detect Nrf2 recognized the epitope on the protein. The addition of the second antibody and conjugated HRP provided a colorimetrically sensitive method of detection by spectrophotometry at a wavelength λ 450 nm in a STATFAX4200 plate reader.

2.5 Statistic analysis

Normality tests and student's t were used for paired samples, whereas; values obtained before and after the tests were analyzed with Pearson's correlation. The data were analyzed using the SPSS 15.0 statistical software.

2.6 Ethical considerations

The study followed the declaration of Helsinki in agreement with national and international laws. This study was approved by the Research Ethics Committee of Hospital General de Durango 450 and by the academic nucleus of the Master's Degree in Health Sciences, FAMEN-UJED, Durango, Dgo. Mexico.

3. Results

This study included participants with a previous DM diagnosis based on the 2018 ADA and NPD (Michigan) criteria. The participants had 15±12 years with diabetes and the mean age of all patients was 58±8 years. Of all the patients, only 16% were of average weight; while, the rest were overweight and obese i.e. 32% and 52% respectively. According to the Child-Pugh diagnostic criteria, no evidence of apparent alterations in liver function was reported. While glucose level was found controlled in 32% of participants and the rest did not show adequate control. With respect to the chronic degenerative diseases, 44% participants had dyslipidemia , 52% had arterial hypertension while nephropathy was found in 4% of the patients.

According to ATP III criteria, the components to diagnose metabolic syndrome include waist > 88 cm, triglycerides ≥ 150 mg/dL, HDL < 50 mg/dL, glucose ≥ 100 mg/dL and BP ≥ 130 mg/dL [34]. In this study, 40% participants were presented with two and 32% with three components of the metabolic syndrome. In addition, , the participants showed high values of glycated hemoglobin (HbA1c) as established by the ADA 2022, which indicates lack of glycemic control. Results are shown in Table 1.

Table 1. Biochemical-clinical characteristics of study participants.

	Before (n=25)	After (n=25)
Hb1Ac (%)	8.3±2.3*	8.2±1.8*
Glucose (mg/dL)	150.2±72*	131.6±36.5*
Albumin (g/dL)	3.8±.2	3.8±.3
Triglycerides (mg/dL)	216.31±112.0*	245.25±165.4*
Total Cholesterol (mg/dL)	174±41.2	185±38.7
HDL (mg/dL).	44.5±11.5	43.3±11
LDL (mg/dL).	86.1±21.4	79.26±25.8
Hemoglobin (g/%)	13.4±1.3	13± 1.3
Creatinin (mg/dL)	0.69±0.06	0.70±0.07
BUN (mg/dL)	16.3±5.4	16.2±6.3
Urea (mg/dL)	35.0±11.6	36.2±10.3

Values are expressed as mean±standard deviation.
*Values outside normal range.

The participants had a median of 4.0 years with neuropathy symptoms and amongst these patients, 68% were diagnosed with severe peripheral neuropathy, while the rest had moderate (24%) and mild (8%) form of the disease. Most of the symptoms were observed in lower limbs, with feet (88%) being the leading site of involvement, followed by thighs (52%). Moreover, discomfort in hands and neck Was also observed in 40% and 24% patients respectively.After treatment with ozone, there was an improvement in the patient's symptoms. The participants were classified as having moderate and mild peripheral neuropathy (4 and 96%, respectively). Statistically significant differences were observed in the presence of burning, pain, stitches, numbness, cramps, and the sensation of walking on stones (Fig. 1A). Additionally, this was reflected in the decrease in difficulty in carrying out a physical activity (Fig. 1B). Later, the participants were grouped as patients with glycemic control and patients with poor glycemic control. Greater treatment efficacy with respect to the reduction in symptoms was observed in those who had adequate glycemic control (Fig. 1C and 1D).

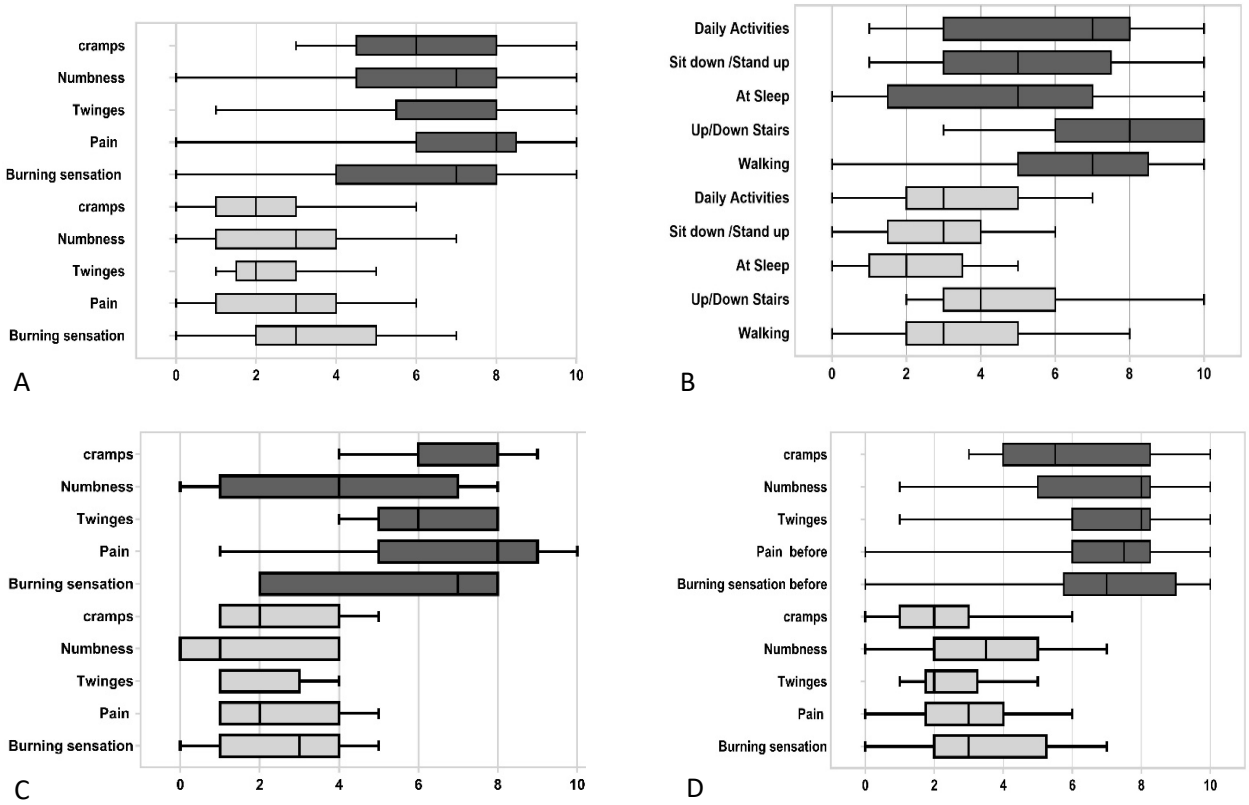

Figure 1. (A) Diabetic peripheral neuropathy symptoms in study subjects, based on the Downie scale ozone treatment. (B) Difficulty in performing daily activities by participants with DNP based on the Downie scale ozone treatment. (C) Difficulty performing daily activities in patients with glycemic control. (D) Difficulty performing daily activities in patients with poor glycemic control.(Dark grey and light grey color represents before and after treatment with ozone respectively).

Before the intervention with ozone, the oxidative status values were: plasma lipoperoxidation: 0.83±0.16 nmol MDA/mL, total antioxidant capacity: 0.249±0.007 Eq. of Trolox [mM] and Nrf2/ARE binding activity: 0.20±0.04.

At the start of treatment, it was found that plasma lipid peroxidation increased and antioxidant capacity decreased, this being proportional to the degree of severity of DNP. After treatment, it reduced plasma oxidative damage by 68% ($p<0.05$).

Table 2. Oxidative status markers in patients with DM.

Oxidative variables	Before (n=25)	After (n=25)	p
Plasma lipoperoxidation (nmol MDA/mL)	0.83±0.16	0.76±0.11	0.015*
Total antioxidant capacity (Eq. de trolox [mM])	0.249±0.007	0.235±0.007	0.037*
Nrf2/ARE binding activity.	0.20±0.04	0.18±0.06	0.013*

*Statistically significant ($p<0.05$), according to the Wilcoxon signed-rank statistical test. MDA: Malondialdehyde. Nrf2/ARE: Nuclear erythroid factor type 2, elemental antioxidant response

The antioxidant capacity was also found to be decreased ($p<0.05$), however, this hasn't biological plausibility. Nevertheless, oxidative damage versus antioxidant capacity observed less dispersion after treatment than before treatment (Fig.2).

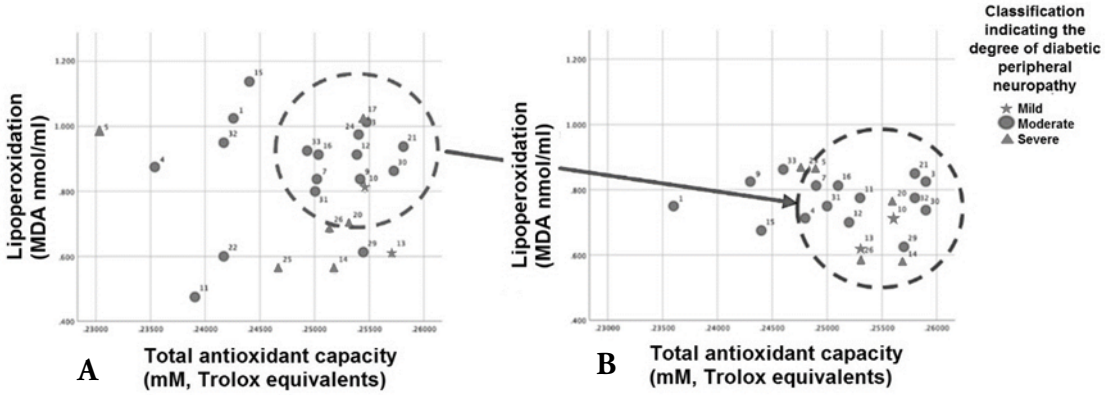

Figure 2. Plasma oxidative damage versus plasma antioxidant capacity (A)Before and (B) after ozone treatment, according to the degree of diabetic peripheral neuropathy.

Regarding HbA1c, at the beginning of treatment, it had a negative correlation with oxidative damage in plasma, before ($r= -0.339$) and after ($r= -0.350$) treatment ($p<0.05$). This suggests that the lower the amount of glycated hemoglobin HbA1c, the lower the oxidative damage, both before and after ozone treatment.

Based on the number of metabolic syndrome components mentioned above, initial evaluation showed that the participants who were only diagnosed with DM presented a higher range of oxidative damage. As the number of components of the metabolic syndrome increased, oxidative damage also increased. After ozone treatment, a decrease in oxidative damage was observed in the plasma of patients only diagnosed with DM and those suffering from two and three components of metabolic syndrome (Fig. 3). The Nrf2/ARE binding activity showed a 10% decrease after ozone treatment i.e. 0.20±0.04($p=0.013$).

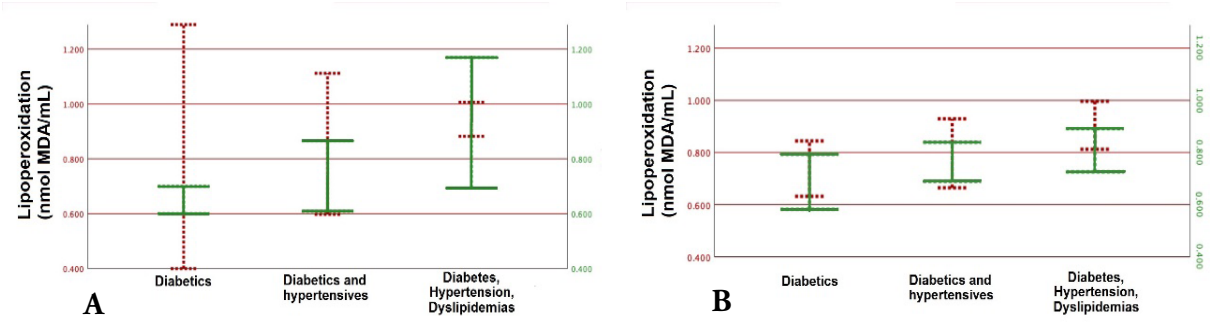

Figure 3. Lipoperoxidation vs. components of the metabolic syndrome. (A) Glycemic control (B) Poor glycemic control before (dotted line) and after (line) ozone treatment.

4. Discussion

DM can damage the peripheral nervous system and DNP is its most common complication and one of the main leading cause of higher rate of morbidity and mortality among diabetic patients.[36-39] In Mexico, DNP is about 50% prevalentin DM patients, after 25 years of evolution, show a.[40] Another study in 2012 reported the occurrence of 69% DNP in peripheral neuropathy patients with DM.. However, the degree of severity was not described.[41] In this study, most of the patients with DNP (92%) were in a moderate and severe stage. Likewise, 84% of patients were obese or overweight.Several studies have reported that obesity, overweight and DNP are risk factors for developing higher intensity of pain and numbness.[42-45] This was consistent with the high prevalence of the severity of symptoms in patients with moderate and severe DNP in this study. Additionally, it was observed that most of the patients in this study suffered from dyslipidemia and arterial hypertension. It has been shown that these comorbidities are associated with a high risk of macro and microvascular complications. In addition, risk for developing clinical complications also increases such as ulceration in lower limbs which can cause amputation, decrease in productive work time, poor quality of life and early deaths.[46]

Oxidative stress is one of the biochemical mechanisms that has been proposed to explain the development and clinical complications related to DM. When the oxidative damage to lipids was evaluated in the study population, lower levels of MDA and fasting blood glucose were obtained after the intervention as compared to the initial values ($p<0.05$). Similarly, negative correlation ($p<0.05$) between lipoperoxidation and HbA1c was also observed. Several studies have suggested that HbA1c is a predictive factor for the development of peripheral polyneuropathy, and it's related to plasma MDA levels in such a way that the higher the HbA1c, the higher the MDA values.[47] The present study confirmed that higher levels of MDA are correlated with intensity of symptoms before treatment which were decreased at the end of the treatment. Likewise, in a study, diabetic foot patients were given adjuvant treatment with local (ozonated gel) and systemic ozone (mixture of ozone and oxygen), rectally or intravenously. Fasting glycemia and plasma lipoperoxidation were decreased significantly in these patients.[26]

As previously mentioned, the participants in this study presented atleast two criteria for the metabolic syndrome (MS) diagnosis as reported by National Cholesterol Education Program Adult Treatment Panel III (NCEP-ATP III). According to this, the adults with MS must meet atleast three of the following criteria i.e. waist in men be >102 cm and >88 cm in women, blood pressure of 130/85 mmHg, HDL <40 mg/dL in men and <50 mg/dL in women, triglycerides >150 mg/dL and fasting glucose >100 mg/dL. This pattern was observed by a study which showed that the greater the number of associated components of the metabolic syndrome, the higher the significance of oxidative damage. This suggests that the sum of different chronic-degenerative diseases favors the overproduction of reactive species perpetuating the hypertension, dyslipidemia, diabetes and its complications in these patients.[48] This result has a biological plausibility because it has been described that products of lipid peroxidation increase not only in patients with prediabetes and DM but also, in those with clinical complications such as retinopathy, kidney damage and DNP.; This suggests the presence of cellular stress and a systemic oxidative state.[18,49] This disruption of the redox state could contribute to the micro and macrovascular changes that occur in DM.

In current study, the antioxidant capacity was found to be decreased after treatment ($p<0.05$). The values obtained were even lower than those reported in other pathologies such as chronic degenerative diseases.[18,36,47,48,50] In recent years, ozone therapy has proven effective in numerous pathologies.[22,28,32,51] Some studies have also evaluated the mechanism of action of this therapy through cellular adaptability processes such as determining the binding activity of Nrf2/ARE which is found to be decreased after ozone treatment. In this respect, a study on chronic diabetic patients, both with controlled and uncontrolled glycemia, reported a statistically significant decrease in Nrf2/ARE binding activity as compared to clinically healthy subjects and patients diagnosed with prediabetes.[47] Another study reported that patients with long-standing diabetes mellitus have less activity of this protein complex when compared with prediabetes. This suggests that the Nrf2/ARE binding activity increases in acute oxidative distress events and decreases according to evolution time, with further decrease in those patients without adequate glycemic control.[52] A possible explanation would be the depletion of Nrf2 due to constant oxidative distress.

Patients with diabetes mellitus and diabetic peripheral neuropathy have a high basal oxidative aggression which increases the severity of the symptoms of this pathology and the therapeutic management that it implies. In turn, baseline antioxidant buffer systems are decreased, favoring a state of oxidative distress. Moderate oxidative stress caused by ozone decreases the activation of the transcriptional factor that mediates Nrf2 related to erythroid factor 2. The Nrf2 domain is responsible for activating the transcription of antioxidant response elements (AREs); due to the ARE, transcription induction decreases and various antioxidant enzyme concentration levels decrease too in response to transient oxidative stress from ozone.[35,53-55] Different mechanisms of action of ozone have been described by various studies. One of these is that ozone upregulates the expression levels of Hsp70 which in turn, is strictly related to HO'. The HO isoforms are recognized as dynamic sensors of cellular

oxidative stress and regulators of redox homeostasis across the phylogenetic spectrum.[56] While Hsp70 is involved in co- and post-translational folding, quality control of misfolded proteins, folding and assembly of de novo proteins in macromolecular complexes, anti-aggregation, protein re-folding and degradation.[57,58] The ozone action is omnidirectional, ranging from immunoregulatory and anti-inflammatory properties to antioxidant activity, antimicrobial effect, analgesic and vasodilator role. Moreover, it is also involved in promotion of blood flow and oxygenation, regenerative processes modulator and epigenetic modifications. Therefore, this study confirms the benefits of the therapeutic use of ozone and contributes to the existing knowledge of the molecular mechanism of oxidative damage so that it can be used in other pathologies whose oxidative state is altered.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare no conflict of interest.

Acknowledgement

The authors gratefully acknowledge the generosity of Edgar Rios and Dennis Quintanar who participated in this research, in addition to thanking the National Science and Technology Council for awarding a scholarship to Angel Saul Almaguer Blanco.

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