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Medical ozone promotes Nrf2 phosphorylation reducing oxidative stress and pro-inflammatory cytokines in multiple sclerosis patients

Livan Delgado-Roche[a,*], Mario Riera-Romo[a], Fernando Mesta[b], Yanet Hernández-Matos[c], Juan M. Barrios[d], Gregorio Martínez-Sánchez[e], Said M. Al-Dalaien[f]

[a] Department of Pharmacology, Institute of Marine Sciences, Havana 10600, Cuba
[b] Instituto de Ciencias Biomédicas, Universidad Autónoma de Ciudad Juárez, Ciudad Juárez 32315, Chihuahua, Mexico
[c] Pharmacy and Food Science Institute, University of Havana, Havana, Cuba
[d] Laboratory of Oxidative Stress, Mexican Association of Oxidative Stress, Mexico D.F., Mexico
[e] Medical Center Beauty Benefit – San Biagio di Osimo, Via Mons. Oscar Romero, 31, 60027 Osimo, Ancona, Italy
[f] Department of Pharmacology, Medicine College, Mutah University, Jordan

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ABSTRACT

Oxidative stress and inflammation play key roles in the pathogenesis of Multiple sclerosis (MS). Different drugs have been used in the clinical practice, however, there is not a completely effective treatment. Due to its potential therapeutic action, medical ozone represents a promising approach for neurodegenerative disorders. The aim of the present study was to address the role of ozone therapy on the cellular redox state in MS patients. Ozone (20 μg/ml) was administered three times per week during a month by rectal insufflation. The effect of ozone therapy on biomarkers of oxidative stress and inflammation was addressed by spectrophotometric and immunoenzymatic assays. Furthermore, we investigated the action of ozone on CK2 expression and Nrf2 phosphorylation by western blotting analysis. Medical ozone significantly improved ($P < 0.05$) the activity of antioxidant enzymes and increased the levels of cellular reduced glutathione. In accordance, a significant reduction ($P < 0.05$) of oxidative damage on lipids and proteins was observed in ozone-treated patients. As well, the levels of pro-inflammatory cytokines TNFα and IL-1β were lower after ozone treatment. Ozone therapy incremented the CK2 expression together with Nrf2 phosphorylation in mononuclear cells of MS patients. These findings suggest that ozone´s antioxidant and anti-inflammatory effects might be partially associated with an induction of Nrf2 phosphorylation and activation. These results provide new insights on the molecular events modulated by ozone, and pointed out ozone therapy as a potential therapeutic alternative for MS patients.

1. Introduction

Neurodegenerative disorders (NDs) are a group of heterogeneous diseases of the nervous system, including the brain, spinal cord, and peripheral nerves that have many different etiologies. The incidence of NDs increases with extended life expectancy, representing a serious health problem worldwide (Milo et al., 2014). Multiple sclerosis (MS) is a chronic inflammatory autoimmune disease of the central nervous system (CNS) that leads to demyelination and axonal damage (Lisak, 2007; Gonsette et al., 2008; Amedei et al., 2012). In MS patients, focal lesions of white and gray matter frequently coincide with extensive cortical demyelination (Frohman et al., 2006). MS is classified in four clinical categories, Relapsing-remitting MS (RRMS) which represent at least 85% of cases; Secondary progressive MS, Primary progressive MS and Progressive-relapsing MS are the other categories. Clinical symptoms course can change indistinctly and so the patient shift from one classification to another (Milo et al., 2014).

In addition to inflammation, oxidative stress (OS) has been strongly associated with the pathogenesis of MS (Lev et al., 2006; Lutskii et al., 2007; Pentón-Rol et al., 2011). The overproduction of reactive oxygen species damage essential cellular components in the nervous tissue, including lipids, proteins and nucleic acids (Gilgun-Sherki et al., 2004). High levels of lipid peroxidation and decreased antioxidants have been observed in blood and cerebrospinal fluid of patients at active phases of MS (Greco et al., 1999; Ferretti et al., 2005). Moreover, reactive oxygen species promote transendothelial leukocyte migration and contribute to oligodendrocyte damage and axonal degeneration. In turn, activated leukocytes produce pro-inflammatory cytokines and reactive oxygen species (Frohman et al., 2006).

The expression of some antioxidant enzymes is tightly regulated by

* Corresponding author.
E-mail address: ldelgadoroche@gmail.com (L. Delgado-Roche).

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the transcriptional factor NF-E2-related factor 2 (Nrf2) (van Horssen et al., 2010). The Nrf2 transcriptional activity is regulated by the Kelch-like ECH associated protein1 (Keap1) in the cytosol, where it acts as redox sensor (Itoh et al., 2004; Yamazaki et al., 2015). Reactive oxygen species or other electrophiles suppress Keap1-dependent Nrf2 degradation leading to Nrf2 nuclear translocation, where it binds to electrophile-responsive element (EpRE) of genes coding for antioxidant enzymes (Itoh et al., 2010; Canning et al., 2015). The casein kinase 2 (CK2) have been identified as another regulator of the Nrf2 activity through its phosphorylation (Pi et al., 2007; Apopa et al., 2008). A number of literature reports suggest that Nrf2 pharmacological activation confers neuroprotective effects in experimental models of NDs (Nakaso et al., 2006; Neymotin et al., 2011; Magesha et al., 2012; Petri et al., 2012; Arnold et al., 2014).

Up to now, some disease-modifying therapies have been introduced to the clinical practice for relapsing/remitting MS treatment, including immunosuppressive, immunomodulatory and neuroprotective agents (Wiendl et al., 2008; Bjelobaba et al., 2016; Doshi and Chataway, 2016). However, the limited clinical efficacy of current drugs justifies the investigation of new therapeutic strategies, including the regulation of redox-sensitive signaling pathways.

The ozone-oxidative conditioning concept (León et al., 1998) supports the pharmacological effect of medical ozone in different OS-associated diseases. The interaction of ozone with serum and cellular components leads to the formation of ozone peroxides, acting as second messengers that activate different pathways associated with cellular redox responses (Sagai and Bocci, 2011; Martínez-Sánchez et al., 2012; Re et al., 2014). Neuroprotective actions have been predicted for ozone based on its biological effects in vivo (Sagai and Bocci, 2011), however less is known on its clinical and therapeutic efficacy in neurodegeneration. A recent report showed the in vivo activation of Nrf2 in peripheral blood mononuclear cells by low doses of ozone and the promotion of protein synthesis in healthy subjects (Re et al., 2014). Based on this evidence; the aim of the present work was to study the effect of medical ozone on the cellular redox status and the levels of pro-inflammatory cytokines in MS patients. Furthermore, we explored the potential action of ozone treatment on Nrf2 phosphorylation, an essential event during the activation of the Nrf2/ARE signaling pathway and the subsequent expression of antioxidant mechanisms.

2. Material and methods

2.1. Medical ozone generation

Ozone was generated from medical grade oxygen by Ozomed Plus equipment (CNIC, Havana, Cuba), representing only about 3% of the gas mixture (O_3/O_2). Ozone was administered by rectal insufflation immediately upon generation. 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 and the International Scientific Committee in Ozone Therapy ISCO3 (ISCO3, 2014).

2.2. Study design

Adult patients of both gender and different ethnic origin with a diagnosis of RRMS, which were not at exacerbation episode of the disease, were included in the study. All patients gave their informed consent to participate in the trial. Exclusion criteria were the presence of exacerbation of the disease, any non-communicable chronic disease, severe septic conditions, and hypersensitivity to medical ozone, hepatic dysfunction, renal failure, and pregnancy, recent history of alcohol or drug abuse and current therapy with any investigational drug or participation in other clinical study. As control group, 40 age-matched healthy subjects (20–50 years old) were also included. This clinical study was carried out in accordance with the principles of the Declaration of Helsinki (WMA, 2013).

Patients (n = 28) were treated with 20 μg/ml of ozone in 50 ml as total volume, three times per week during a month, as recommended previously (Viebahn-Hänsler et al., 2012). Nelaton catheter (Visa Laboratory S.A. de C.V., Mexico) was used to deliver the gas into the rectum for 5 min. The patients were encouraged to empty the bowel before the procedure.

2.3. Plasma collection for biochemical determinations

Blood samples were obtained in heparinized tubes after a 12 h overnight fast, before the treatment and 24 h after the last dose of ozone. The samples were immediately centrifuged at 3000*g*, for 10 min, at 4 °C. Then, the plasma was collected and aliquots were stored at −80 °C until analysis.

2.4. Quantification of pro-inflammatory and anti-inflammatory cytokines

The serum levels of pro-inflammatory cytokines interleukin 1-beta (IL-1β) and the tumor necrosis factor alpha (TNFα) were determined by ELISA kits (Pharmingen, USA), the detection limits were 0.5 and 0.12 pg/ml, with intra-assay variation of 2.8% and 2.5%, respectively. The anti-inflammatory interleukin 10 (IL-10) levels were quantified by high-sensitivity ELISA (R & D Systems, USA), the detection limit was 0.5 pg/ml, and intra-assay variation among triplicates was 7.8%. In case where cytokine levels are not detectable, the lower limit of detection will be assigned as the theoretical value.

2.5. Oxidative stress biomarkers

All redox parameters were examined by spectrophotometric methods using a Spectrophotometer Gensys 6 (Thermoscientific, USA). Superoxide dismutase (SOD) activity was determined by using a commercial kit (Sigma-Aldrich, USA) according to the manufacturer instructions. Catalase (CAT) activity was assessed by following the decomposition of hydrogen peroxide (H_2O_2) at 240 nm for 1 min. The activity of Glutathione peroxidase (GPx) was recorded following the oxidation of NADPH at 340 nm as previously described (Paglia et al., 1967). Meanwhile, glutathione-S-transferase (GST) activity was determined using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. The assay was initiated with addition of plasma (50 μl) in a reaction mixture containing potassium phosphate buffer (0.3 M, pH 6.5), reduced glutathione (30 mM), and CDNB (30 mM). The reaction was continuously monitored for 5 min at 340 nm. The enzymatic activity was expressed in nmol of CDNB-GSH conjugates formed/min (Habig et al., 1974). After precipitation of thiol proteins, the reduced glutathione (GSH) levels were measured according to the method of Sedlak and Lindsay with Ellman's reagent (5,5`dithiobis-2-nitrobenzoic acid) (Sigma, USA) at 412 nm (Sedlak and Lindsay, 1968). The advanced oxidation protein products (AOPP) were quantified as previously described (Witko-Sarsat et al., 1998). Briefly, 50 μl of plasma were treated with 25 μl of 1.16 M potassium iodide followed by the addition of 50 μl of acetic acid. The absorbance was immediately read at 340 nm. AOPP concentration was expressed as μM of chloramines-T. Concentration of malondialdehyde (MDA) was determined using the LPO-586 kit obtained from Calbiochem (La Jolla, CA, USA).

2.6. Cell isolation and culture

Mononuclear cells (MNC) were isolated from heparinized blood by Ficoll density gradient centrifugation (Ficoll Plus, eBiosciences). The cells were resuspended and 3×10^4 cells/ml were cultured in RPMI medium containing 10% (v/v) fetal bovine serum (FBS) and 1% penicillin-streptomycin (Life Technologies, USA) at 37 °C in 5% CO_2 humidified incubator. Four h later, MNC were treated for western

blotting analysis.

2.7. Analysis of CK2 expression and Nrf2 phosphorylation by western blotting

Cell lysates were obtained using a lysis buffer (0.02% aprotonin, 2% SDS, 10% glycerol, 62.5 mM Tris-HCl, and 20 mM sodium fluoride, pH = 6.8) on ice for 15 min. Lysates were centrifuged at 2000*g* for 15 min at 4 °C. Then, equal amounts of protein (50 μg) were fractionated in 10% SDS-polyacrylamide gel. Primary antibodies that recognizes the phosphorylated and non-phosphorylated form of Nrf2 (Santa Cruz Biotechnology Inc.), the caseine kinase-2α (Abcam), and β-actin (Abcam) were used. The membranes were incubated with diluted antibodies (following manufacture instructions), overnight at 4 °C with gentle shaking. After washing three times with PBS-Tween 20%, the membranes were incubated with a secondary anti-rabbit antibody (1:2500) conjugated to horseradish peroxidase (Promega) for 1 h at room temperature. Finally, protein bands were visualized with DAB and quantitative data normalized against β-actin by densitometry analyses performed using the *GeneTools* program (Syngene).

2.8. Statistical analysis

Values were expressed as mean ± standard error of the mean (S.E.M.). Statistical analysis was performed with SPSS 12.0 software. For multiple comparisons, one-way ANOVA was used followed by Bonferroni post-hoc test. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Baseline clinical, and demographic characteristics of the studied population

In relation to the baseline characteristics of studied population (Table 1), both groups were similar at randomization ($p > 0.05$). In both groups, more than 40% of subjects were older than 31 years and females were the majority. The medical history of MS patients was characterized mainly by neuropathic pain (57%), sensitive-motor disorders (32%), and optic neuritis (11%). The presence of smoking (32%) was detected as a risk factor for MS progression. No side effects were observed or reported in patients involved in the study. No relevant differences between men and women were found in a preliminary data analysis, thus the results were analyzed globally. The 81% of patients received 6 MUI of interferon beta-1a (Rebif®, Merck) subcutaneously, three days per week, meanwhile the 19% only consumed a natural antioxidant combination based on Spirel® (*Spirulina platensis*) and Vimang® (aqueous extract of *Maginfera indica*). During ozone treatment, the antioxidant therapy was interrupted, because of the potential interference between antioxidants and ozone. However, INFβ therapy was not interrupted.

Table 1
Baseline clinical and demographic characteristics of the studied population.

Parameters	MS patients (n = 28)	Controls (n = 40)
Age (years)		
20–30	6 (21%)	9 (23%)
31–40	12 (43%)	16 (40%)
41–50	10 (36%)	15 (37%)
Gender		
Females	21 (75%)	24 (60%)
Males	7 (25%)	16 (40%)
Risk factors		
Smokers	9 (32%)	–
Clinical history		
Neuropathic pain	16 (57%)	–
Sensitive-motor disorders	9 (32%)	–
Optic neuritis	3 (11%)	–

Legend: No significant statistical differences between groups ($P > 0.05$) for demographic variables were achieved. The diagnosis and diseases' manifestations were recorded as previously reported by the clinicians in patients' clinical history.

Table 2
Effect of ozone therapy on pro- and anti-inflammatory cytokines.

Cytokines levels (pg/ml)	Control group (n = 40)	MS patients before ozone therapy (n=28)	MS patients after ozone therapy (n=28)
IL-1β	1.89 ± 0.27[a]	6.04 ± 0.85[b]	3.68 ± 0.46[c]
TNF-α	2.08 ± 0.19[a]	4.82 ± 0.89[b]	2.15 ± 0.13[a]
IL-10	14.10 ± 1.28[a]	5.71 ± 1.63[b]	9.84 ± 1.26[c]

Legend: Values are means ± SEM of three independent experiments. The levels of IL-1β, TNFα and IL-10 were determined in plasma samples of MS patients (n = 28) before and after the ozone treatment by ELISA methods. Cytokines were also measured in plasma samples of control healthy subjects (n = 40). Ozone treatment decreased the serum levels of pro-inflammatory cytokines together with an increment of the anti-inflammatory IL-10. In healthy subjects where cytokine levels were not detectable, the lower limit of detection was assigned as the theoretical value. This happened in 8/40 for IL-1β, 5/40 for TNF-α, and 11/40 for IL-10 of controls. Different letters represent statistical differences (ANOVA-Bonferroni post-test, $P < 0.05$). MS: multiple sclerosis, IL-1β: interleukin-1beta, TNF-α: tumor necrosis factor-alpha, IL-10: interleukin-10.

Due to the analgesic effects of medical ozone (Borrelli et al., 2011; Magalhaes et al., 2012; Bocci et al., 2015), patients where asked about neuropathic pain relieve after a week of the last dose of ozone. From the patients that where asked about their symptoms relieve, 87% of them (14/16) said the pain sensitivity was reduced after treatment, significantly in legs and arms.

3.2. Ozone treatment decreases TNFα, IL-1β, and incremented IL-10 serum levels

To evaluate the effect of ozone treatment on inflammation, we measured the plasma levels of TNFα, IL-1β and IL-10 in MS patients. As shown in Table 2, after ozone treatment the serum levels of pro-inflammatory TNFα and IL-1β cytokines significantly diminished ($P < 0.05$), meanwhile a significant increment ($P < 0.05$) of the anti-inflammatory IL-10 was observed.

3.3. Ozone treatment modulate oxidative stress

To address the regulatory effect of ozone therapy on OS, we measured the activity of the antioxidant enzymes SOD, GPx, CAT and GST as well as the GSH levels, a non-enzymatic antioxidant. In addition, we measured MDA and AOPP, as surrogate markers of lipid and protein oxidation, respectively. The results are shown in Table 3. We detected a significant increase ($P < 0.05$) of antioxidant enzymes activity after ozone treatment. On the other hand, ozone insufflation significantly ($P < 0.05$) increases the concentration of GSH, together with a reduction of MDA and AOPP levels in MS patients. These results demonstrated that ozone therapy modulates the antioxidant/pro-oxidant balance in MS patients, enhancing antioxidant mechanisms and reducing the lipid and protein oxidative damage.

3.4. CK2 expression and Nrf2 phosphorylation are enhanced by ozone treatment

In order to determine if ozone treatment affects the expression of CK2 and Nrf2 phosphorylation, we performed a western blot analysis in mononuclear cells from MS patients. As shown in Fig. 1, the phosphorylated form of Nrf2 is increased in the patients after ozone treatment. In addition, an enhancing of CK2 expression after ozone treatment was observed (Fig. 1). These data indicate that ozone therapy is able to promote the Nrf2 phosphorylation, through a mechanism that

Table 3
Effect of medical ozone administration on biomarkers of oxidative stress.

Biomarker	Control group (n=40)	MS patients before ozone therapy (n=28)	MS patients after ozone therapy (n=28)
SOD (U/l)	291.00 ± 18.04 [a]	219.76 ± 20.31 [b]	723.51 ± 29.32 [c]
GPx (U/l)	160.25 ± 20.72 [a]	81.45 ± 9.33 [b]	172.32 ± 26.78 [a]
CAT (U/l)	1127.00 ± 45.61 [a]	753.00 ± 24.78 [b]	1331.00 ± 38.74 [c]
GST (nmol/min)	23.49 ± 4.58 [a]	14.66 ± 2.39 [b]	22.70 ± 5.11 [a]
GSH (μmol/l)	8.21 ± 1.66 [a]	3.93 ± 1.21 [b]	11.35 ± 1.86 [c]
MDA (μmol/l)	1.80 ± 0.68 [a]	7.59 ± 1.07 [b]	3.46 ± 0.91 [c]
AOPP (μmol/l)	6.14 ± 0.79 [a]	17.69 ± 1.43 [b]	12.44 ± 1.26 [c]

Legend: Values are means ± S.E.M. of oxidative stress biomarkers. Ozone treatment improved the antioxidant status, reducing lipid and protein oxidative damage. Different letters represent statistical differences (ANOVA-Bonferroni post-test, P < 0.05). MS: multiple sclerosis, SOD: superoxide dismutase, GPx: glutathione peroxidase, CAT: catalase, GSH: reduced glutathione, GST: glutathione-S-transferase, MDA: malondialdehyde, AOPP: advanced oxidation protein products.

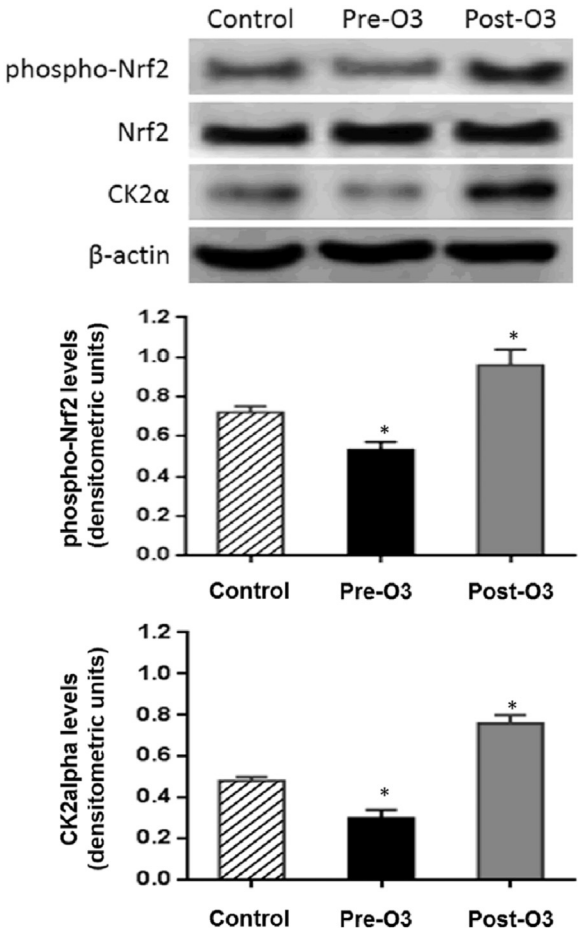

Fig. 1. Detection of Nrf2 phosphorylation and CK2 expression in mononuclear cells. Mononuclear cells were isolated from heparinized blood of MS patients (n = 28) and control healthy subjects (n = 40). The cells from blood samples of patients were isolated before and after the ozone treatment. Total cell extracts (50 μg) were fractioned by SDS-PAGE and immunoblotted with anti-CK2α and anti-Nrf2 antibodies. β-actin was measured as a control for loading variations. The bands were analyzed by densitometry. Values represent mean ± S.E.M. of three independent experiments (*P < 0.05).

involves, at least, the overexpression of the kinase CK2.

4. Discussion

Ozone has been described as a toxic and irritating gas, particularly for the respiratory tract (Pryor et al., 1995). However, this toxicity depends entirely on ozone concentration, time of exposure as well as the antioxidant and neutralizing capabilities of the biological system. It has been demonstrated that a small and precisely calculated dose of ozone administered by systemic routes for a few minutes, displays important pharmacological effects increasing the systemic antioxidant status (Sagai and Bocci, 2011). According to the ozone oxidative preconditioning concept, a regulated exposure of a medium with antioxidant defenses (like blood, tissues) to a low dose of ozone triggers a small and transient oxidative stress that ensures medical efficacy, but no toxicity (León et al., 1998).

Despite the skepticisms, preliminary data suggest that low doses of ozone may be effective in the treatment of different chronic conditions related with inflammation and OS, such as diabetes (Martínez-Sánchez et al., 2005), coronary artery disease (Martínez-Sánchez et al., 2012), chronic obstructive pulmonary disease (Borrelli and Bocci, 2014), atherosclerosis (Delgado-Roche et al., 2014), and rheumatoid arthritis (Dranguet et al., 2013; León et al., 2016). Medical ozone can be delivered by rectal insufflation, which constitutes a simple and minimally invasive route, with experimentally demonstrated low toxicity, representing an alternative to the classical mayor auto-hemotherapy (MAH). Furthermore, the biological effects of the rectal insufflation of ozone has been demonstrated extensively either experimentally or clinically (Martínez-Sánchez and Re, 2012). Thus, in the present study we used the rectal route to achieve a systemic delivery of ozone.

The health benefits of ozone therapy include an increase of systemic antioxidants, a reduction of oxidative damages, an improvement of oxygen blood transportation and delivery, cytoprotection and anti-inflammatory effects (Re et al., 2014). Because of its anti-inflammatory and antioxidant inducing capacity, ozone therapy is a plausible alternative to treat NDs (Sagai and Bocci, 2011). A previous study demonstrated the neuroprotective action of ozone in a rat model of Parkinson's disease (Re et al., 2008). However, there are not concluding evidences from clinical studies. Furthermore, a clinical trial involved MS patients demonstrated an increment of cytochrome-c-oxidase level induced by ozone autohemotherapy, revealing a reduction of the chronic oxidative stress level typical of MS sufferers (Molinari et al., 2014).

Chronic pain is a common symptom of neurologic disease, including MS. The persistent and progressive neuropathic pain in MS patients is a life-altering condition that greatly affects the quality of life (Khan et al., 2014). Pain is a subjective experience and pain measurements must rely on the patients' self-report. In this study, patients report a reduction of the painful sensitivity after a first cycle of ozone treatment. Previous reports confirmed that ozone administration at low doses reduces spinal pains, such as cervical pain and low back pain (with or without disc herniation-related sciatic pain) (Borrelli, 2011; Pecorelli et al., 2013). As suggested by Bocci and coworkers, ozone can display a number of analgesic effects, ranging from the reduction of inflammation, correction of ischemia and venous stasis to finally inducing a reflex therapy effect by stimulating anti-nociception analgesic mechanisms (Bocci et al., 2015). In addition, ozone can regulate the expression of the genes that play a pivotal role in the onset and maintenance of allodynia (normalized the mRNA caspase-1, caspase-12 and caspase-8 gene levels, but did not the decrease caspase-3 level) and also reduced IL-1β (Fuccio et al., 2009).

Inflammation plays a central role in the pathogenesis of MS, which include T cell-mediated autoimmune reactions and pro-inflammatory cascades amplified by cytokines (Lisak, 2007). Here, we demonstrated that ozone therapy decreases the circulating levels of TNFα and IL-1β that are strongly associated with MS progression (Amedei et al., 2012). Both cytokines are activators of NF-κB transcription factor, resulting in a downstream cascade of pro-inflammatory cytokine synthesis, contributing with a persistent chronic inflammation (Flodströma et al., 1996). On the other hand, low levels of anti-inflammatory cytokines have been observed in animal models and in MS patients (Amedei

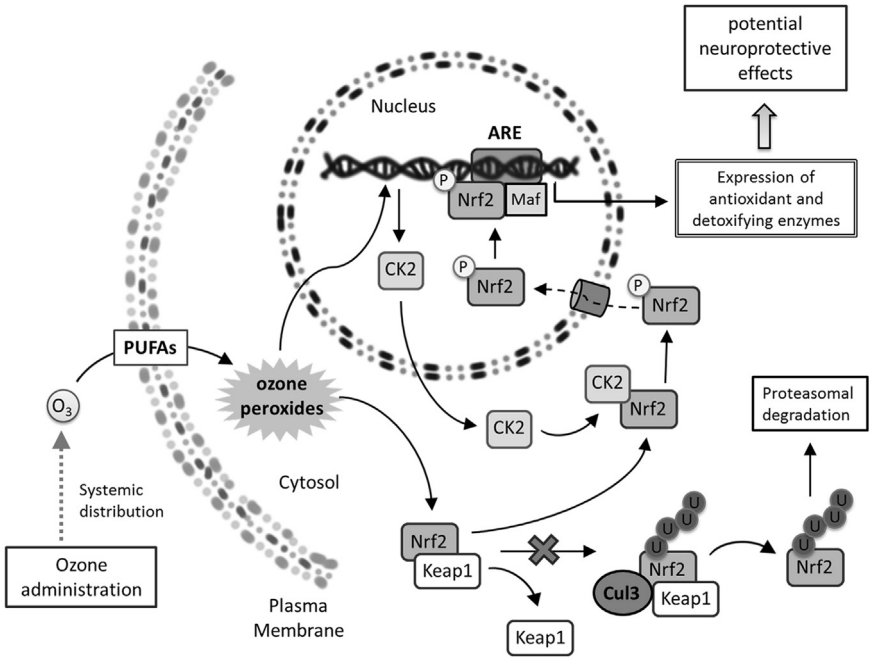

Fig. 2. Hypothetic effects of ozone mediators on Nrf2 activation in mononuclear cells from MS patients. After its administration the ozone rapidly dissolves and reacts with the polyunsaturated fatty acids (PUFAs) of plasma membrane producing different hydroxy-hydroperoxides, known as "lipid ozonated products". Once inside the cell, these second messengers increase the expression of Casein kinase 2 (CK2) possibly acting at a nuclear level. They also dissociate the complex between the NF-E2-related factor 2 (Nrf2) and the Kelch-like ECH-associated protein 1 (Keap1), releasing Nrf2 and preventing its Cul3-dependent ubiquitination and proteasomal degradation. The free cytosolic Nrf2 is phosphorylated by CK2, what leads to its nuclear translocation and transcriptional activation. Activated Nrf2 in a complex with Maf proteins binds to the electrophile-responsive element (EpRE) promoting the expression of different antioxidant and detoxifying enzymes that mediate neuroprotection.

et al., 2012; Trenova et al., 2017). Literature data showed that INF-β (Nicoletti et al., 2000; Ersoy et al., 2005; Kieseier, 2011; Haji et al., 2016) and glucocorticoids (Gayo et al., 1998; Krieger et al., 2014; Goodin, 2014), an accepted disease-modifying agents for MS therapy, not only reduce the pro-inflammatory cytokines but also induced the increment of serum anti-inflammatory cytokines. Here we noted an increment of IL-10 serum levels in ozone-treated patients, which suggest an immune system regulation and anti-inflammatory effects of medical ozone in MS patients. The immunomodulatory effect of medical ozone has been characterized by the increased serum levels of INF-γ and INF-β, as well as other cytokines such as IL-2, IL-6 and IL-8 (Sagai and Bocci, 2011). Recent data showed a protective effect of medical ozone against ethidium bromide-induced demyelination in rats, either alone or in combination with low doses of corticosteroids. A synergistic anti-inflammatory effect for corticosteroids combining ozone therapy was observed, together with a reduction of lipid peroxidation and an improvement of brain antioxidants (Salem et al., 2016). The anti-inflammatory effect of medical ozone has been observed in other chronic inflammatory disorders, including rheumatoid arthritis (Dranguet et al., 2013; Borrelli and Bocci, 2014; León et al., 2016). Recently, our group demonstrate that medical ozone increased methotrexate clinical response in patients with rheumatoid arthritis, reducing pro-inflammatory cytokines and improving cellular redox balance (León et al., 2016). In a recent future, the combined therapy based on disease-modifying agent plus ozone may represent an alternative which not only improve the efficacy of traditional drugs but also reduce their toxicity.

On the other hand, due to its capacity to modulate redox pathways and the expression of antioxidant defenses, the neuroprotective potential of ozone has been predicted previously (Re et al., 2008; Sagai and Bocci, 2011). The present results demonstrated that ozone therapy improves the antioxidant status and reduces the oxidative damage in MS patients, which might be associated with the promotion of Nrf2 phosphorylation. A low transcriptional activity of Nrf2 has been related with the development of experimental autoimmune encephalomyelitis (EAE), an experimental model of MS (Nguyen et al., 2009). In addition, it is well known that Nrf2 activity is regulated by CK2 through direct phosphorylation (Pi et al., 2007). Some studies have revealed that ozone treatment increases the nuclear localization of Nrf2 (Pecorelli et al., 2013). Furthermore, in 2014, Re and coworkers reported an increase of Nrf2 activity by ozone oxidative pre-conditioning in healthy subjects (Re et al., 2014). However, in the mentioned study, the molecular basis of this activation was not clarified.

The nuclear localization and transcriptional activity of Nrf2 are regulated by two independent mechanisms within the cell: 1) the protein Keap1 that inhibits Nrf2, impairing its nuclear translocation and promoting its Cul3-dependent degradation (Itoh et al., 2004; Magesha et al., 2012) and 2) the MAP kinase CK2 that regulates the transcriptional activity and nuclear localization of Nrf2 through phosphorylation (Apopa et al., 2008). Different electrophiles such as tert-butylhydroquinone, DL-sulforaphane, lipoic acid, curcumin, hydroperoxides and dimethyl fumarate are able to dissociate the Keap1-Nrf2 interaction via oxidation of cysteine residues of Keap1, an event that promote the dissociation from Nrf2 (Magesha et al., 2012). One of these Nrf2 inducers, the tert-butylhydroquinone, also shows the capacity to promote the CK2-mediated phosphorylation of Nrf2 in human neuroblastoma cells with the subsequent transcriptional activity (Khan et al., 2014). These results suggest the Nrf2 dissociation from Keap1 as a necessary step for the subsequent phosphorylation, activation and nuclear translocation.

Ozone acts as pro-drug, because it rapidly disappears after reaction within the organism, generating two second messengers: H_2O_2 and a mixture of lipid ozonated products (LOP) that results from the reaction of ozone with the cell membrane and albumin- or lipoproteins-bound polyunsaturated fatty acids (PUFAs). The hydroxy-hydroperoxides (known as "ozone peroxides") have been postulated to be pharmacologically active LOP. These second messengers oxidize the cysteine residues (Viebahn-Hänsler et al., 2012), which might promote Keap1-Nrf2 dissociation and activation of Nrf2-ARE pathways (See Fig. 2). In the present work, we demonstrated by western blot analysis, that ozone

treatment increases the expression of CK2 and the subsequent Nrf2 phosphorylation. The phosphorylated form of Nrf2 localizes preferentially in the nucleus and becomes transcriptionally active (Apopa et al., 2008), which could explain the increment of antioxidant enzymes after ozone treatment. This finding is consistent with previous studies, which demonstrate that Nrf2 activation is associated, in part, with CK2-induced phosphorylation (Apopa et al., 2008; Kim et al., 2012; Pecorelli et al., 2013).

A deficient transcriptional activity of Nrf2 is strongly related to the pathogenesis of experimental autoimmune encephalomyelitis (EAE), a murine model of MS (Nguyen et al., 2009). As consequence of Nrf2 deficiency, the induction of gene encoding detoxifying and antioxidant enzymes, including hemeoxygenase-1 (HO-1) (Itoh et al., 2004). A reduced expression of HO-1 have been associated with the disease activity and severity in MS patients (Fagone et al., 2013; Jernås et al., 2013; Agúndez et al., 2016). It has been reported that EAE induction in HO-1 deficient mice is more severe than in HO-1 wildtype animals, being partially reversed by administration of carbon monoxide, the resultant product of HO-1 activity (Chora et al., 2007; Fagone et al., 2011). Prophylactic administration of carbon monoxide donors partially improves clinical and histopathological features in rodent EAE models (Fagone et al., 2011). Despite the potential therapeutic role for HO-1 and carbon monoxide in MS (Fagone et al., 2012; Wilson et al., 2017), there are limited clinical evidences. On the other hand, experimental Nrf2 activators have been evaluated in animal models as candidates for MS treatment. A recent study demonstrated that TFM-735, a potent Nrf2 inducer, inhibits inflammatory cytokines production and disease progression in mice with EAE (Higashi et al., 2017). In the clinical scenario, Dimethyl fumarate (Tecfidera®), an up-regulator of the Nrf2 pathway (Canning et al., 2015), has been approved by the European Union and the US Food and Drug Administration (FDA) for the treatment of RRMS (Gopal et al., 2017; Havrdova et al., 2017). These evidences further support that pharmacological modulation of Nrf2 activity as a therapeutic approach for NDs. However, the main disadvantage of some Nrf2 activators is the poor penetration across the blood-brain-barrier (Petri et al., 2012; Ghadiri et al., 2017). In contrast, ozone therapy constitutes a pharmacological alternative based on a high permeable agent, which might combines the potential dissociation of the Keap1-Nrf2 interaction with the enhancing of CK2 expression, two crucial events necessary for the phosphorylation of Nrf2 and its transcriptional activity (see Fig. 2).

5. Conclusions

In summary, ozone therapy promotes a reduction of cellular oxidative stress as reflect the increment of antioxidant enzymes activity and the reduction of oxidative damage on lipid and proteins. In addition, medical ozone reduced the pro-inflammatory cytokines levels together with a reduction of serum IL-10, an anti-inflammatory cytokine. The regulatory effect of medical ozone was partially associated with the induction of Nrf2 phosphorylation. Further investigations are required to corroborate the role Nrf2 as target of ozone mediators in MS disease. The impact of ozone therapy on Keap1-mediated inhibition of Nrf2, as well as the events associated with ozone therapy-induced CK2 expression should be further explored in experimental models (e.g. cell cultures and EAE animal models with Nrf2 deficiency). These studies will provide further insights to suggest the potential neuroprotective mechanism of medical ozone in MS. In addition, a future phase II clinical trial should be addressed to validate the efficacy of medical ozone as a complementary therapy for MS patients. Finally, our results suggest that ozone therapy may be used to potentiate the efficacy of disease-modifying agents, permitting the use of low doses of these drugs together with a reduction of toxicity and side effects.

Conflict of interests

The authors have no conflicts of interests to report.

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