

RESEARCH ARTICLE

The effects of oxygen–ozone therapy on regulatory T-cell responses in multiple sclerosis patients

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Abstract

Multiple sclerosis (MS) is a common degenerative disorder of the central nervous system. The decreased frequency and dysfunction of Treg cells cause inflammation and disease progression. Ozone autohemotherapy can be used as a potential therapeutic approach to regulate the immune system responses and inflammation in MS. For this purpose, 20 relapsing-remitting multiple sclerosis patients were under treatment with ozone twice weekly for 6 months. The frequency of Treg cell, the expression levels of the Treg cell-related factors (*FoxP3*, *IL-10*, *TGF-β*, *miR-17*, *miR-27*, and *miR-146A*), and the secretion levels of *IL-10* and *TGF-β* were assessed. We found a significant increase in the number of Treg cells, expression levels of *FoxP3*, miRNAs (*miR-17* and *miR-27*), *IL-10*, and *TGF-β* factors in patients after oxygen–ozone (O_2-O_3) therapy compared to before treatment. In contrast, oxygen–ozone therapy notably decreased the expression level of *miR-146a* in treated patients. Interestingly, the secretion levels of both *IL-10* and *TGF-β* cytokines were considerably increased in both serum and supernatant of cultured peripheral blood mononuclear cells in posttreatment condition compared to pre-treatment condition. According to results, oxygen–ozone therapy raised the frequency of Treg cell and its relevant factors in treated MS patients. Oxygen–ozone therapy would contribute to improving the MS patients by elevating the Treg cell responses.

KEYWORDS

cytokines, microRNAs, multiple sclerosis, oxygen–ozone therapy, transcription factor, Treg cell

Abbreviations: CNS, central nervous system; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; FoxP3, forkhead box P3; IRAK1, interleukin 1 receptor-associated kinase 1; miRNAs, microRNAs; MS, multiple sclerosis; NIRS, near-infrared spectroscopy; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; RRMS, relapsing-remitting multiple sclerosis; STAT1, signal transducer and activator of transcription 1; TGF-β, transforming growth factor-beta; TNF-α, tumor necrosis factor-alpha; TRAF6, TNF receptor-associated factor 6.

1 | INTRODUCTION

Multiple sclerosis (MS) is identified as a chronic neurodegenerative disorder of the central nervous system (CNS) and is clustered as a common autoimmune disease. Mostly, MS involves the 20–40 years old individual and causes neurological, physical, and cognitive disturbances (Compston & Coles, 2008; Frohman et al., 2006). Both genetics and environmental risk factors are associated with disease pathogenesis, in which class II haplotype HLA-DRB is the most strongly relevant allele to MS (Ahmadi, Gharibi et al., 2017; Aslani et al., 2017). The cross-talk between the blood-brain barrier and autoreactive lymphocytes is considered as an essential etiology of MS occurrence that entailed the axonal loss, demyelination, and gliotic scarring due to local inflammation of entered lymphocytes (Dolati, Ahmadi et al., 2018). Based on immunological studies, increased levels of Th1 and Th17 cells, higher levels of proinflammatory cytokines, dysfunction of Treg cell, and the presence of autoreactive B cells, are the substantial reasons correlated with MS progression. However, it has been evidenced that T-cell responses and their inflammatory products against myelin antigens have central role in MS pathogenesis by degenerating the myelin sheaths of central neurons (Danikowski et al., 2017; Høglund & Maghazachi, 2014).

Treg cell expressing the CD25, CD4, and forkhead box P3 (*FoxP3*) markers is the regulatory subtype of CD4+ T cells. Immunologically, Treg cell has the main role in secreting the transforming growth factor-beta (TGF- β) and IL-10 cytokines, suppressing the autoreactive T cells, regulating the immune responses against infectious or cancers, and maintaining the immunologic self-tolerance (Braithc et al., 2009). IL-10, as an anti-inflammatory cytokine, has an immune regulatory function. It has a critical role in suppressing the inflammatory cytokines and a protective role in autoimmunity. *FoxP3* and TGF- β are the essential factors required for Treg cell differentiation from naive T CD4+ cells. TGF- β promotes Treg cell development by inducing the expression of *FoxP3*. Treg cell has a pivotal suppressive function contributing to autoimmune disease improvement like MS. Accordingly, Treg cell dysfunction in MS, followed by the self-tolerance breakdown, is led to autoreactive T-cell suppression failure, myelin and neural destruction, and neuroinflammation (Bjerg et al., 2012). Prominently, in most autoimmune diseases, the ratio of Th17 and Treg cells determines the progression or protection outcome of disease. Evidently, it has been shown that the imbalanced levels of Th17 and Treg cells, as well as the Treg cell dysfunctionality, result in MS development (Cong et al., 2016; Jamshidian et al., 2013).

Treg cell regulation is performed by a *FoxP3* transcription factor and several diverse microRNAs (miRNAs). miRNAs, as single-stranded RNAs involved in MS pathogenesis, are the posttranscriptional epigenetic regulatory factors containing the 18–25 base-pair noncoding RNAs (Alipour et al., 2017; Bartel, 2004). miRNAs are introduced as pluripotent agents contributing to the development, differentiation, and proliferation of cells, as well as inflammation, apoptosis, metabolism, and angiogenesis by regulating the

expression of target genes. Also, they can be employed as prognostic and diagnostic biomarkers in several disorders (Kanwar et al., 2010; Sonntag, 2010). Various dysregulated miRNAs, including miR-17, miR-27, and miR-146a, are considerably associated with MS pathogenesis and its biological process (Dolati, Aghebati-Maleki et al., 2018; Dolati et al., 2019). These miRNAs can change the presentation of target antigens and regulate the responses of immune cells like Treg cells. Ozone (O₃) gas, as a water-soluble inorganic molecule, is formed of three cyclic structure oxygen atoms (V. A. Bocci, 2006; Elvis & Ekta, 2011). Encouragingly, it has been evidenced that ozone would be a beneficial therapeutic agent in various disorders due to the antioxidant and antibacterial capabilities (V. Bocci et al., 2009), induction of oxidative stress tolerance, and prevention of free radical damage (León et al., 1998). Based upon previous studies, ozone demonstrated the efficient potential in improving the chronic infections of antibiotic-resistant pathogens, vascular diseases (V. Bocci et al., 2011), vascular ulcers (Shah et al., 2011), diseases of the jaw (Elvis & Ekta, 2011), and neurologic disorders like MS (Lintas et al., 2013). The purpose of the current study was to assess the ozone autohemotherapy (O₃-AHT) in MS patients by evaluating the alteration of Treg cell frequency and its relevant factors (*FoxP3*, IL-10, TGF- β , miR-17, miR-27, and miR-146a) before and after treatment.

2 | MATERIAL AND METHODS

2.1 | Study design and population

The current study was performed based on a randomized, double-blind way with doing the randomization according to a computer-generated list. Also, the randomization code and allocation of the group were done blinded to the participants and hospital staff. The 6-month randomized trial study was piloted on an overall 20 patients with relapsing-remitting multiple sclerosis (RRMS) confirmed by diagnosing the clinical symptoms before participating in our study. Also, history, physical and neurological conditions, brain magnetic resonance imaging, and laboratory tests were examined in patients before initiating the intervention. In addition, we enrolled 20-matched healthy control subjects to investigate the considered parameters at baseline and in comparison with patients. Written informed consent was taken from all included patients and healthy controls and the study was approved by the Ethics Committee of Baqyatallah University of Medical Sciences (IR.BMCU.REC.1398.041). Oxygen-ozone therapy was applied to each patient as a treatment approach. Blood sampling of patients was conducted one time before oxygen-ozone therapy and the second time after treatment. The inclusion criteria of the study were the satisfaction of patients to participate in the study, MS diagnosis based on clinical symptoms, and MacDonald criteria. Also, the exclusion criteria were as follows: patients should not be Bedridden, not be pregnant, and not be over 75 years old, and have not any other autoimmune or

TABLE 1 Demographic of the MS-treated patients with ozone therapy and controls

Parameters	Ozone-treated patients group	Healthy control group	p Value
Number	20	20	NS
Sex, male/female, %	20/0 (100)	20/0 (100)	NS
Age (min-max), years	33.6 ± 5.21 (23-42)	34.08 ± 4.49 (27-46)	NS
BMI	26.58 ± 5.23	25.98 ± 4.98	NS
Familial history	1	None	-
EDSS	1.18 ± 0.39	-	-
Disease duration (min-max), years	4.2 ± 1.5 (2-8)	-	-

Abbreviations: BMI, body mass index; MS, multiple sclerosis.

infectious diseases, thyroid dysfunction, coagulation and platelet disorders, hypocalcemia, and any neurological, metabolic, or cardiovascular diseases as well as sensitivity to ozone. All patients were fasting and were followed by a therapy consisting of beta interferon injections and corticosteroids administered in correspondence of attacks and the patients had an inadequate response to these drugs. Demographic information and clinical features of MS patients and healthy controls have been listed in Table 1.

2.2 | Oxygen-ozone therapy protocol

Medical ozone is an ozone/oxygen mixture consisting of purest O₂ and purest O₃ produced from medical oxygen by a medical ozone generator. Major autohemotherapy with ozone was conducted by collecting 100 ml of venous blood from the median cubital vein of MS patients into a heparinized vacuum flask, an extracorporeally closed sterile system, where the ozone/oxygen gas mixture is poured to it before being reinfused. Ozone maker (HAB Herrmann Apparatebau GmbH) was used to mix the O₂ with O₃. Then, to generate the ozonized blood, 100 ml of heparinized blood was incubated with 100 ml of O₂/O₃ mixture (25 µg ozone/ml ozone/oxygen gas mixture) for 3-5 min with gentle movement. The ozonized blood was slowly reinfused into patients by the same median cubital vein, following passed through a sterile filter (Viebahn-Hänsler et al., 2012). Oxygen-ozone therapy administration was performed 25 times twice per week, and the experiment was taken under precise medical observation.

2.3 | Blood collection, peripheral blood mononuclear cell separation, and cell culture

Blood collection from the healthy control group and the patient group was performed before and after 6-month oxygen-ozone therapy. 10 ml of heparinized whole blood samples were collected. The peripheral blood mononuclear cells (PBMCs) were separated based on density-gradient centrifugation through

1.077 g/ml standard Ficoll (Lymphosep) (Biosera). After that, PBMCs were centrifuged at 450 g for 25 min and washed by phosphate-buffered saline (PBS) (Sigma-Aldrich) twice. The separated cells (5×10^6) were cultured in a 5 ml culture medium comprising the 10% heat-inactivated fetal bovine serum (FBS), 200 mM L-glutamine, 100 U/ml penicillin, and 10 ng/ml phorbol myristate acetate (eBioscience). Culture plates were incubated at 37°C and 5% CO₂ for 48 h. Finally, the cultured cells were gathered to use for measuring the messenger RNA (mRNA) gene expression by real-time polymerase chain reaction (PCR), and supernatant of them was utilized for assessing the concentration of cytokines by enzyme-linked immunosorbent assay (ELISA).

2.4 | Real-time PCR

Real-time PCR measured the expression levels of *FoxP3*, *TGF-β*, *IL-10*, and miRNA (*miR-17*, *miR-27*, and *miR-146a*) genes along with specific forward and reverse primers and SYBR green approach. After cell culture, total RNA extraction from PBMCs was implemented using the RNX-PLU solution (SinaClon). Then, Revert Aid Reverse Transcriptase Kit (Thermo Fisher Scientific) was utilized to synthesize the complementary DNA from extracted RNA. Next, mRNA expression was detected based on the standard steps of real-time PCR, as follows: (1) The denaturation phase with 40 repeated cycles at 95°C for 10 s; (2) The annealing step at 58°C (*FoxP3*), and 60°C (*TGF-β* and *IL-10*) for 30 s; (3) The extension step at 72°C for 20 s. The verification of gene amplification was performed by electrophoresis analysis on 2% agarose gel and DNA sequencing by Biosystems (Seqlab). To obtain the standard curves, a concentrated sample of the genes was used to provide the six standards by serial dilutions. Finally, the PCR reactions were done utilizing the SYBR Green master mix. U6 small nuclear and β-actin were considered as housekeeping RNA controls. Data analysis was conducted in a 2^{-ΔΔC_t} manner to assess the expression relative of target genes to the housekeeping genes and normalize the expression folds of mentioned target genes. The sequences of primers have been listed in Table 2.

TABLE 2 Sequences of primers

Gene	Primer	Sequence
TGF- β	Forward	CGACTACTACGCCAAGGA
	Reverse	GAGAGCAACACGGGTTCA
IL-10	Forward	CAT CGA TTT CTT CCC TGT GAA
	Reverse	TCTTGGAGCTTATTAAGGCATTC
FoxP3	Forward	TCATCCGCTGGGCCATCCTG
	Reverse	GTGGAAACCTCACTTCTTGCTC
miR-17	Forward	GAGCCAAAGTGCTTACAGTGC
	Reverse	AGTGCAGGGTCCGAGGTATT
miR-27	Forward	CAGTTCACAGTGGCTAAGA
	Reverse	CAGTTTTTTTTTTTTTTCGGGAA
miR-146a	Forward	GCCGCCCTGTGAAATTCAGTT
	Reverse	GTGCAGGGTCCGAGG
β -Actin	Forward	AGAGCTACGAGCTGCCTGAC
	Reverse	AGCACTGTGTGGCGTACAG
RNU6	Forward	CTCGCTTCGGCAGCACATATACT
	Reverse	ACGCTTCACGAATTTGCGTGTC

2.5 | Determination of TGF- β and IL-10 levels by ELISA

The secretion levels of TGF- β and IL-10 produced cytokines by Treg cell, were detected in the supernatant of cultured PBMCs and serum samples of subjects using an ELISA kit (MyBioSource) based on the manufacturer's protocol. In brief, 100 μ l of coating antibody was used to coat the 96-well plate overnight. Then, the plate was washed by PBS, comprising the Tween-20 (0.05%). Next, incubation was done using the blocking buffer to block the empty wells. 100 μ l of samples and standards were added to wells, incubated for 1 h, and then washed. 100 μ l of biotinylated antibody were added to wells and incubated for 1 h. Procedure continued by pouring 100 μ l of the avidin-biotin-peroxidase complex and incubation for 30 min. Thereafter, the plate was washed and 100 μ l of tetramethylbenzidine, as the substrate solution, was added into wells. Eventually, the reaction was stopped after 20–25 min, and the absorbance values of wells were read at 450 nm using the Medgenix ELISA reader (BP-800; Biohit).

2.6 | Assessment of Treg cell frequency by flow cytometry

The flow cytometry method was used to investigate the frequency of Treg cells (CD4⁺ CD25⁺ CD127⁻). To stain cell surface, fluorescein isothiocyanate-labeled anti-human CD4, phycoerythrin-labeled anti-human CD25, and PerCP-conjugated anti-CD127 monoclonal antibodies (eBioscience) were exploited to incubate with PBMCs

(1×10^6) at 4°C for 15 min. The FACS Calibur flow cytometer (BD Biosciences) and Flowing Software 2.4.1 were used to analyze the stained cells.

2.7 | Statistical analysis

SPSS PC Statistics (version 19.0; SPSS Inc.) was used for statistical analysis. Findings were presented as mean \pm SD along with $p < .05$ as statistically significant. The unpaired t-test was used to compare the statistical discrepancy of measured immunological markers before and after oxygen-ozone therapy. To test the linear trend among immune variables, variables response rate, and treatment results, the linear-by-linear association the χ^2 test were implemented.

3 | RESULTS

3.1 | Assessment of FoxP3 expression level

Real-time PCR measured the expression level of FoxP3 transcription factor in MS patient group and healthy control group. Our results revealed a significantly lower expression level of FoxP3 in patients than in controls (0.763 ± 0.341 vs. 0.996 ± 0.091 , $p = .005$) (Figure 1a). To investigate the effect of oxygen-ozone therapy, we also assessed the expression level of FoxP3 in the pretreatment and posttreatment conditions of patients. It was found that the expression level of FoxP3 was significantly increased after oxygen-ozone therapy compared to baseline (2.08 ± 1.123 vs. 1.009 ± 0.118 , $p = .002$) (Figure 1b).

3.2 | Expression levels of miRNAs (miR-17, miR-27, and miR-146a)

First, real-time PCR quantified the expression levels of Treg cell-associated miRNAs (miR-17, miR-27, and miR-146a) in patients and controls. As results, the meaningfully increased expression levels of miR-17 and miR-27 and notably decreased expression level of miR-146a were obtained in MS patients compared to healthy individuals (1.876 ± 0.841 vs. 1.002 ± 0.083 , $p = .0004$; 2.015 ± 0.817 vs. 1.001 ± 0.071 , $p < .0001$; and 0.76 ± 0.303 vs. 1.008 ± 0.075 , $p = .004$, respectively) (Figure 2a). Then, we compared the expression levels of mentioned miRNAs in patients before and after treatment with ozone. Findings indicated the considerable reduction in the expression levels of miR-17 and miR-27 after treatment with ozone versus before treatment (0.750 ± 0.257 vs. 1.001 ± 0.071 , $p = .001$ and 0.662 ± 0.271 vs. 1.001 ± 0.071 , $p = .0004$, respectively). Moreover, O₃-AHT noticeably elevated the mRNA expression level of miR-146a in posttreatment condition than in pretreatment condition (2.376 ± 1.478 vs. 1.001 ± 0.080 , $p = .003$) (Figure 2b).

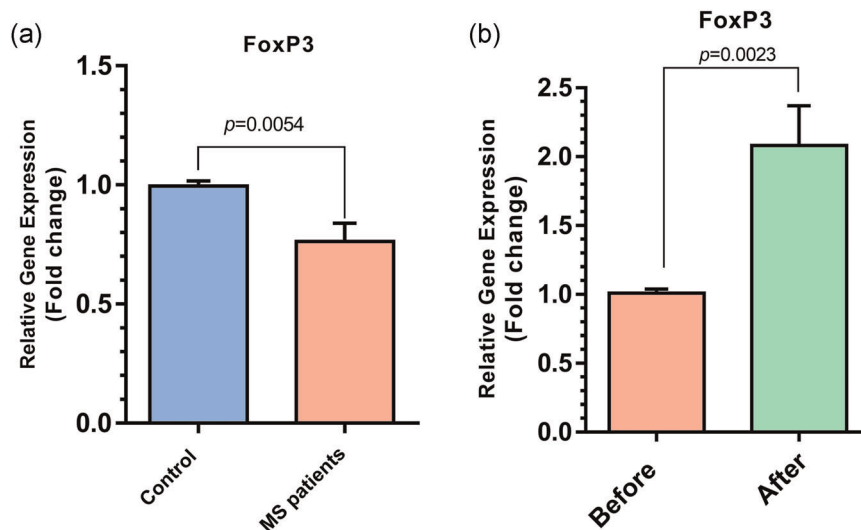


FIGURE 1 The expression level of *FoxP3* in healthy controls and patients before and after oxygen–ozone therapy. (a) In comparison between patient and control groups, the expression of *FoxP3* was at a lower level in MS patients ($p = .0054$). (b) After oxygen–ozone therapy, a significantly increased expression level of *FoxP3* was observed in patients after treatment versus before treatment ($p = .0023$). Ozone-treated group ($n = 20$); control group ($n = 20$). Results were described as mean \pm SD. $p < .05$ was considered statistically significant. FoxP3, forkhead box P3; MS, multiple sclerosis

3.3 | The expression levels of *IL-10* and *TGF- β* cytokines

We detected the expression levels of *IL-10* and *TGF- β* cytokines using real-time PCR in the patient group and controls. As a comparison, the mRNA expression levels of both *IL-10* and *TGF- β* cytokines were found to be considerably lower in MS patients than in healthy controls (0.743 ± 0.252 vs. 1.038 ± 0.128 , $p = .001$ and 0.822 ± 0.218 vs. 1.018 ± 0.105 , $p = .007$, respectively) (Figure 3a). Furthermore, the expression levels of both mentioned cytokines were tested in MS patients before and after O_3 -AHT. As expected, the oxygen–ozone therapy could significantly enhance the expression levels of both *IL-10* and *TGF- β* cytokines in posttreatment patients compared to pretreatment patients (1.871 ± 1.333 vs. 1.047 ± 0.146 , $p = .02$ and 1.614 ± 0.919 vs. 1.016 ± 0.090 , $p = .012$, respectively) (Figure 3b).

3.4 | Cytokine secretion levels of *IL-10* and *TGF- β* in serum and supernatant of cultured PBMCs

We examined the secretion levels of *IL-10* and *TGF- β* cytokines produced by Treg cells in serum and supernatant of cultured PBMCs by ELISA. At first, the comparison between patient groups and controls revealed the remarkable decrease in secretion level of *IL-10* and no significant difference in secretion level of *TGF- β* , in the serum of patients (28.54 ± 12.92 vs. 40.92 ± 22.34 , $p = .03$ and 53.89 ± 36.7 vs. 78.04 ± 46.5 , $p = .12$, respectively) (Figure 4a). Whereas, we observed a significant decline in secretion levels of both *IL-10* and *TGF- β*

cytokines in the supernatant of cultured PBMCs of patients compared to controls (558.6 ± 353.3 vs. 906.7 ± 529.7 , $p = .019$ and 162.5 ± 82.67 vs. 231.1 ± 88.59 , $p = .015$, respectively) (Figure 4b). To investigate the therapeutic effect of oxygen–ozone therapy, we also evaluated the secretion levels of mentioned cytokines before and after treatment with ozone in patients. Our results demonstrated the significantly elevated cytokine secretion levels of both *IL-10* and *TGF- β* in the serum of MS patients, posttreatment versus pretreatment (46.81 ± 25.92 vs. 28.54 ± 12.92 , $p = .034$ and 94.97 ± 49.65 vs. 53.89 ± 36.70 , $p = .014$, respectively) (Figure 4c). Interestingly, assessing the *IL-10* and *TGF- β* secretion levels in the supernatant of cultured PBMCs showed that both of them were notably greater in patients after treatment with ozone versus before treatment (1039 ± 558.4 vs. 558.6 ± 353.3 , $p = .011$ and 299.9 ± 199.8 vs. 162.5 ± 82.67 , $p = .016$, respectively) (Figure 4d).

3.5 | Assessment of the Treg cell frequency

The frequency of Treg cells was assessed by flow cytometry in both patient (before and after oxygen–ozone therapy) and control groups (Figure 5a). The flowcytometric data indicated a significant decline in the frequency of circulating Treg cells in the peripheral blood of MS patients than in controls ($5.172\% \pm 2.29\%$ vs. $7.663\% \pm 2.77\%$, $p = .003$) (Figure 5b). Interestingly, we found that the frequency of Treg cell was remarkably elevated in patients after treatment with ozone compared to the results before treatment ($7.058\% \pm 3.111\%$ vs. $5.173\% \pm 2.298\%$, $p = .035$) (Figure 5c).

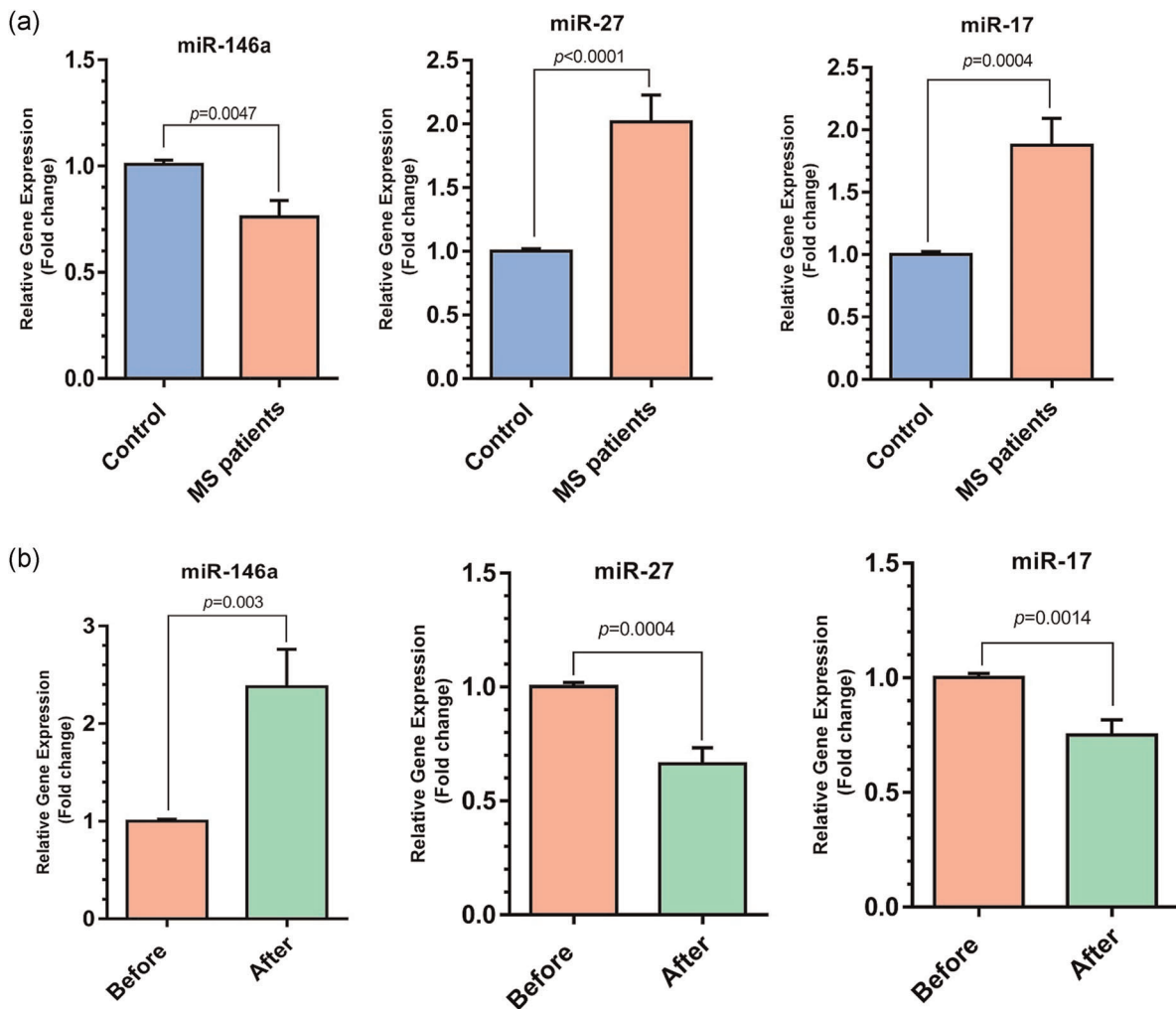


FIGURE 2 Expression levels of Treg cell-related microRNAs in the control group and MS patients in pretreatment and posttreatment conditions with ozone. (a) The considerably higher expression levels of *miR-17* and *miR-27* and lower expression level of *miR-146a* were detected in MS patients than in controls ($p = .0004$, $p \leq .0001$, and $p = .0047$, respectively). (b) After oxygen–ozone therapy results indicated a significant decrease in expression levels of *miR-17* and *miR-27* and a remarkable increase in the expression level of *miR-146a* in patients after treatment when compared to patients before treatment ($p = .0014$, $p = .0004$, and $p = .003$, respectively). MS patient group ($n = 20$); control group ($n = 20$). Results were described as mean \pm SD. $p < .05$ was considered statistically significant. MS, multiple sclerosis; miRNA, microRNA; Treg, T-regulatory

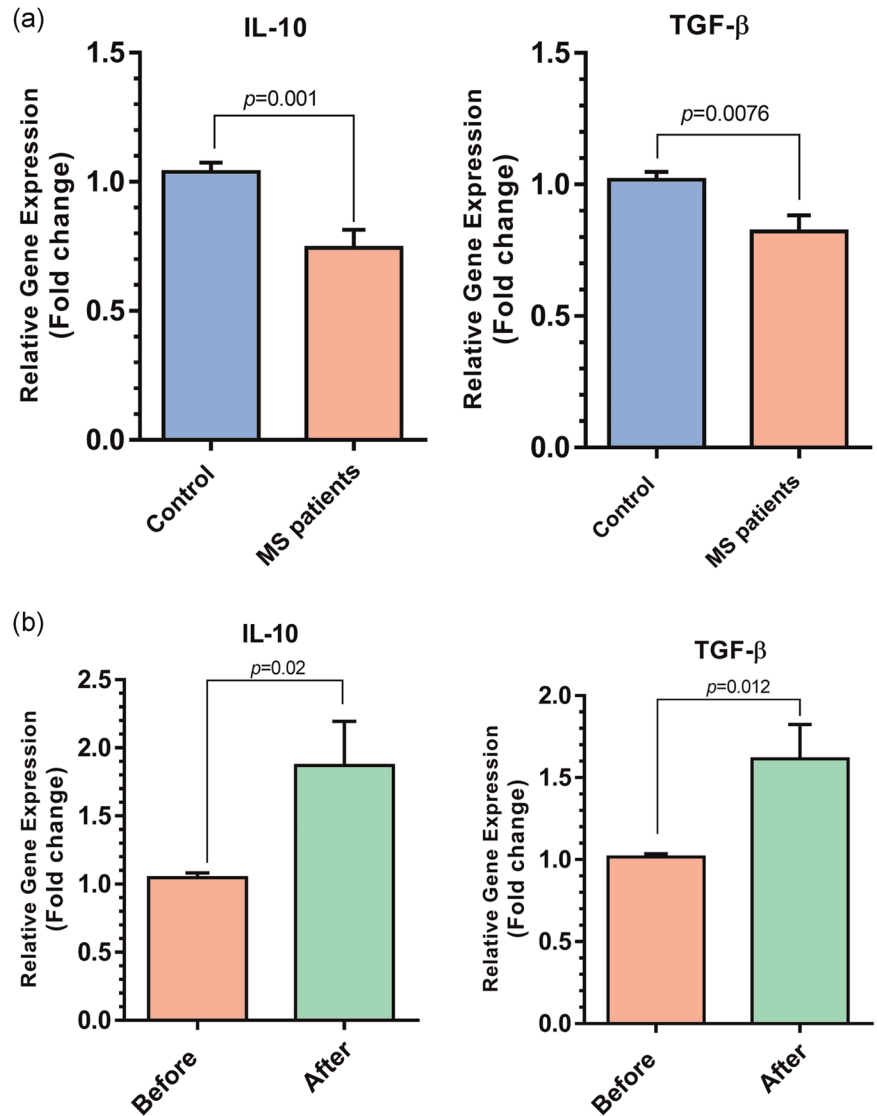
4 | DISCUSSION

Ozone (O_3) is a water-soluble, unstable, and inorganic molecule with transient interaction and mesomeric states. Ozone has multifaceted therapeutic aspects such as vascular, hematological, and immunological modulatory effects. Based on treatment goals, the diverse range of ozone can be administered to patients (10–80 $\mu\text{g}/\text{mL}$ of gas per mL of blood) (Elvis & Ekta, 2011; Zanardi et al., 2016). According to previously published studies, O_3 -AHT has indicated the potential capability and beneficial outcomes in different disorders, including the heart- and cerebrovascular-ischemia, immune deficiency, rheumatoid arthritis, and MS (V. A. Bocci, 2006; Zanardi et al., 2016). Oxygen–ozone therapy can increase the oxygen delivery into the tissues by inducing the red blood cell glycolysis. Also, it can activate the Krebs cycle, which enhances the ATP, decreases the

NADH, contributes to cytochrome C oxidation, and prevents free radicals production (Aydos et al., 2014). As described previously, oxygen–ozone therapy mediates the immune system balance with immunomodulatory effects based on the immune response level and condition of diseases. Moreover, oxygen–ozone therapy can trigger the function of the immune system like cytokine production.

Oxygen–ozone therapy augments the antioxidant system of the body and improves the treatment of diseases (V. A. Bocci, 2006). Ozone upregulates the related enzymes leading to the reduction of oxidative stress (Aydos et al., 2014). Oxygen–ozone therapy also elicits the production of reactive oxygen species and LOP factors, which improve the pain. Increased oxygen delivery (Tylicki et al., 2001), regulating the expression of cytokines and adhesion factors, and improving the glycolytic pathways (Caglayan & Bayer, 1994) are the other advantages of oxygen–ozone therapy.

FIGURE 3 The expression levels of *IL-10* and *TGF- β* cytokines in the control group and MS patients before and after oxygen–ozone therapy. (a) Findings reported the remarkably reduced expression levels of both cytokines in MS patients compared to healthy controls ($p = .001$ and $p = .007$, respectively). (b) Ozone autohemotherapy significantly boosted the expression levels of *IL-10* and *TGF- β* cytokines in patients with the posttreatment condition compared to the pretreatment condition ($p = .02$ and $p = .012$, respectively). MS patient group ($n = 20$); control group ($n = 20$). Results were described as mean \pm SD. $p < .05$ was considered statistically significant. IL, interleukin; MS, multiple sclerosis; *TGF- β* , transforming growth factor-beta



Nevertheless, it has not been clarified whether oxygen–ozone therapy has significant effects on immune cells and factors. Some other related studies have suggested using oxygen–ozone therapy in delaying the aging process and improving neurodegenerative diseases. It has been noted that ozone, as low cost and restricted side effects therapy with anti-inflammation, antioxidant, regenerative, and anti-pathogens features, can be used as a potential therapeutic compound in modulating the immune system, inflammation, microbiota, metabolism, and processes associated with the degenerative process in different disorders (Braidy et al., 2018; Juchniewicz & Lubkowska, 2020; Scassellati, Ciani et al., 2020; Scassellati, Galoforo et al., 2020). However, clear evidenced data about oxygen–ozone therapy efficacy on maintaining the immune system balance did not exist.

Regarding the anti-inflammatory function of oxygen–ozone therapy and its mentioned benefits, in the current study, we evaluated the oxygen–ozone therapy effects on Treg cell and its associated transcription factor (FoxP3), cytokines (IL-10 and TGF- β), and miRNAs (miR-17, miR-27, and miR-146a). This aim was piloted to

improve the inflammation of MS and maintain immune system tolerance.

As previously stated, MS is one of the chronic CNS disorders categorized as an autoimmune-demyelinating disease. Different immunological cells and factors with diverse protective or progressive roles relate to MS pathogenesis or improvement (Compston & Coles, 2008; Ghasemi et al., 2017; Read et al., 2000; Sakaguchi et al., 1985).

Treg cell is the immunosuppressive regulatory cell that has a substantial role in maintaining immunological self-tolerance, in autoimmune and inflammatory conditions (Ahmadi, Abdolmohammadi-Vahid et al., 2017; Sakaguchi et al., 2009, 2008). Immunologically, MS pathogenesis correlates with low frequency and dysfunctionality of Treg cells, increased autoreactive B cells, and dominant responses of Th1 and Th17 cells. Furthermore, an imbalance of Th17/Treg cells has been found as a critical cause of inflammation in inflammatory and autoimmune diseases, leading to disease progression (Ahmadi, Aghdam et al., 2017; Ahmadi et al., 2019; Eghbal-Fard et al., 2019). This may be resulted from the disturbance of immune system

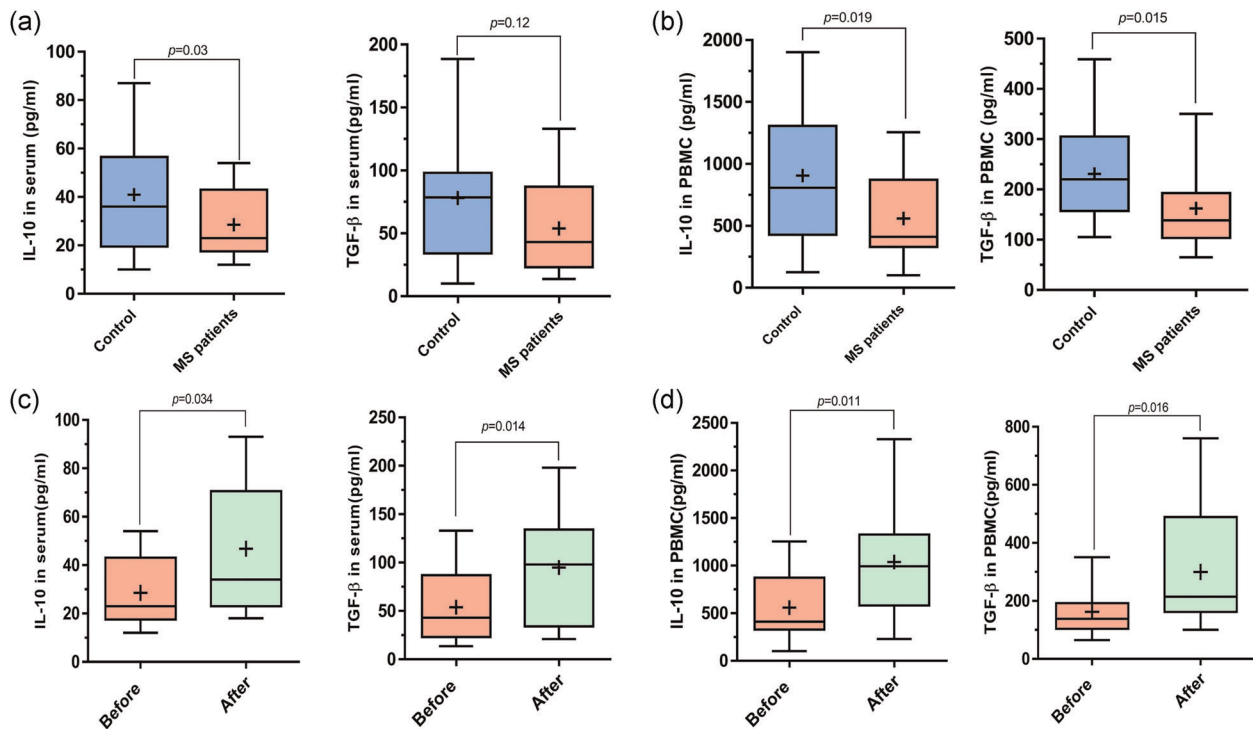


FIGURE 4 The secretion levels of IL-10 and TGF- β cytokines in serum and supernatant of cultured peripheral blood mononuclear cells (PBMCs) in controls and MS patients (pre-and posttreatment with oxygen-ozone therapy). (a) In comparison, the secretion level of IL-10 was remarkably lower in the serum of MS patients than in controls; however, no significant difference was observed in the secretion level of TGF- β in the serum of patients ($p = .03$ and $p = .12$, respectively). (b) The secretion levels of both IL-10 and TGF- β cytokines indicated a notable decline in the supernatant of cultured PBMCs in MS patients compared to controls ($p = .019$ and $p = .015$, respectively). (c) Treatment with ozone leads to the considerable enhancement of IL-10 and TGF- β secretion levels in the serum of treated MS patients in comparison with baseline ($p = .034$ and $p = .014$, respectively). (d) In posttreatment condition with oxygen-ozone therapy, the secretion levels of IL-10 and TGF- β cytokines were found to be considerably greater in the supernatant of cultured PBMCs compared to patients in pretreatment conditions ($p = .011$ and $p = .016$, respectively). IL, interleukin; MS, multiple sclerosis; TGF- β , transforming growth factor-beta

balance and demyelination, causing neuronal destruction and inflammation. It has been reported that, in MS patients, Treg cell frequency and their related cytokines are lower than in healthy individuals (Viglietta et al., 2004). In the current study, we comparatively investigated the frequency of Treg cells in MS patients and healthy individuals. As a consequence, a decreased number of Treg cells was observed in MS patients. Encouragingly, a significant rise in the number of Treg cells was determined in MS patients after oxygen-ozone therapy. Treg cell is identified by possessing the CD4+CD25+FoxP3+ and expressing the TNFR family proteins, CTLA4 checkpoint, and CD95 (Fas), as modulatory factors. Treg cell with suppressive function actively inhibits the autoreactive T cells, provokes tolerance, and maintains immune homeostasis (Pette et al., 1990). Several studies have investigated the immunosuppressive function of Treg cells in autoimmune diseases, including MS, gastritis, thyroiditis, colitis, and insulin-dependent diabetes (Read et al., 2000; Sakaguchi et al., 1985). In the current study, data analysis of assessing the mRNA expression level of FoxP3 demonstrated the significant growth in the expression level of FoxP3 in MS patients after treatment with ozone. The inhibitory function of the Treg cell is connected with the expression level of the FoxP3+ nuclear transcription factor. FoxP3+ expression is at a low level in

activated T CD4+ cells and T CD8+ cells and at a high level in CD4+CD25+ T cells (Wang et al., 2007). Isolated Treg cells from MS patients indicated a decreased level of FoxP3+, relating to the dysfunction of Treg cells (Huan et al., 2005). However, it has not been clarified whether the low level of FoxP3 correlates with the less expression of FoxP3 or the limit frequency of Treg cells. In addition, Treg cell mediates tolerance maintenance by producing the immunosuppressive cytokines, including IL-35, IL-10, and TGF- β (Sakaguchi et al., 2009; Takahashi et al., 2000). In this investigation, we quantified the expression and secretion levels of IL-10 and TGF- β cytokines before and after oxygen-ozone therapy. Significantly, both mRNA expression and secretion levels of IL-10 and TGF- β cytokines were augmented after oxygen-ozone therapy in patients.

IL-10 and TGF- β cytokines have a substantial role in suppressing the immune system responses and related inflammation, contributing to improving autoimmune disorders. In the presence of TGF- β , FoxP3+ expression is induced, leading to the differentiation of naive T CD4+ cells to Treg cells. TGF- β is a regulatory cytokine with an anti-inflammatory role, which its importance has been evidenced in TGF- β deficient mice with autoimmunity (Kulkarni et al., 1993; Lee et al., 2017). Originally, IL-10 inhibits the secretion of inflammatory

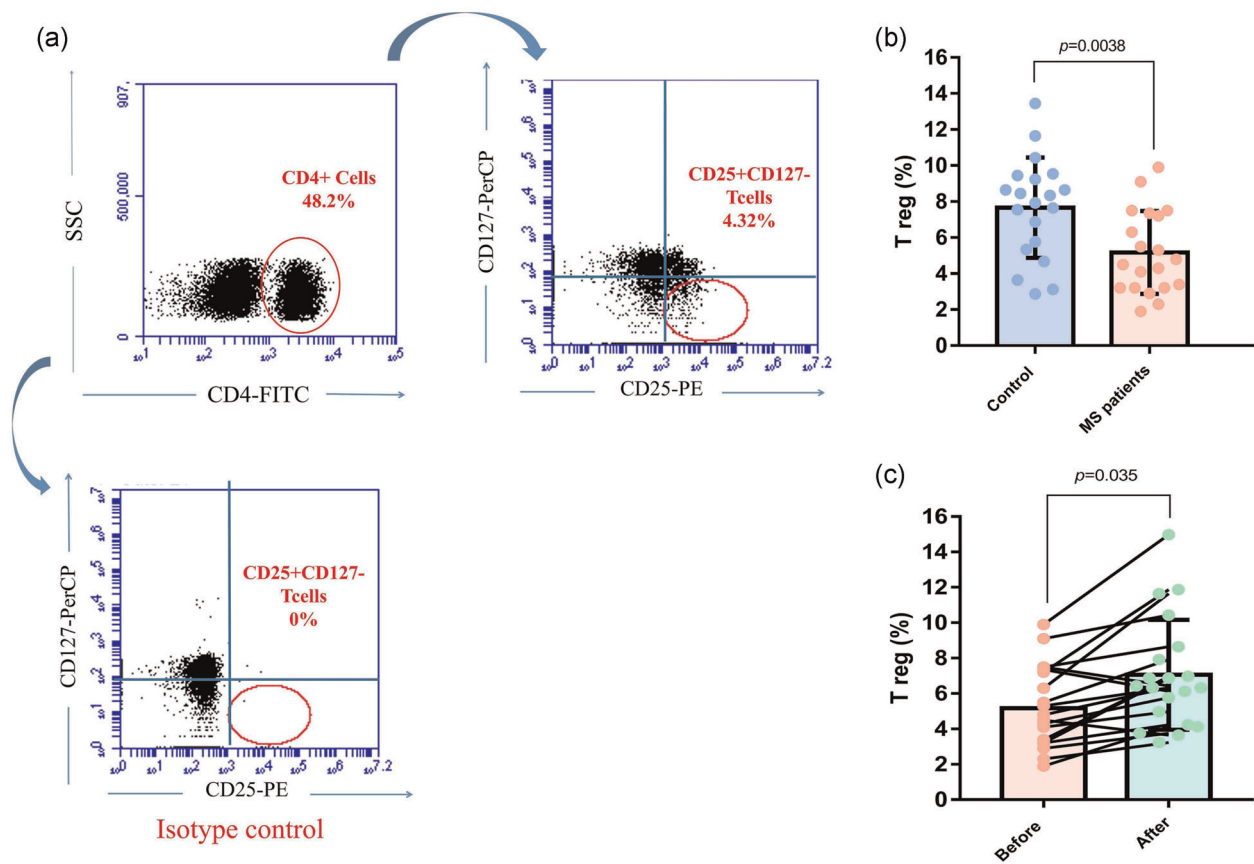


FIGURE 5 Frequency of Treg cell in control subjects and MS patients before and after ozone autohemotherapy. (a) To enumerate the CD4⁺ CD25⁺ CD127⁻ cells, Treg cells were firstly gated according to side scatter and CD4-FITC; then, based on PE-conjugated anti-CD25, and Per-CPconjugated anti-CD127. (b) Flow cytometry data analysis demonstrated a significantly lower frequency of Treg cells in patients compared to controls ($p = .003$). (c) The number of Treg cells showed a prominently increased percentage in patients after oxygen-ozone therapy in comparison with the condition before treatment ($p = .035$). Ozone-treated group ($n = 20$); control group ($n = 20$). Results were described as mean \pm SD. $p < .05$ was considered statistically significant. FITC, fluorescein isothiocyanate; MS, multiple sclerosis; PE, phycoerythrin; PerCP, peridinin-chlorophyll-protein complex; Treg, T-regulatory

cytokines and acts as an immunosuppressive cytokine. The therapeutic capability of IL-10 has been evidenced in various autoimmune disorders, including MS, psoriasis, diabetes, and rheumatoid arthritis (Ozenci et al., 1999). In practice, it has been documented that the decreased levels of IL-10 and TGF- β cytokines in MS patients cause uncontrolled inflammation and disease progression.

miRNAs are the other related factors to MS pathogenesis introduced as endogenous single-stranded noncoding RNA molecules with mRNA gene expression adjusting roles. miRNAs contribute to managing the innate and adaptive immune responses, differentiation of B- and T- cells, and intracellular procedures (Bartel, 2004, 2009). These RNA molecules inhibit the expression of protein-coding genes through the two pathways, including translational suppression and induction of target mRNA cleavage (Gregory et al., 2005). Developing several disorders such as neurodegenerative disorders, autoimmunity, and cancers correlate with the dysregulated expression and function of miRNAs. Dysregulated miRNAs have been reported in various autoimmune diseases, for example, MS, systemic lupus erythematosus, psoriasis, Sjögren's syndrome, and rheumatoid

arthritis. Therefore, the prevention or progression of autoimmune diseases depends on the expression and function of miRNAs (Kanwar et al., 2010; Sonntag, 2010; Tufekci et al., 2011). In the current study, we measured the expression levels of Treg cell-related miRNAs (*miR-17*, *miR-27*, and *miR-146a*) to compare their alteration in patients before and after oxygen-ozone therapy. As a result, significant and nonsignificant decrease in the expression levels of *miR-17* and *miR-27*, respectively, as well as a meaningful increase in the expression level of *miR-146a* were detected in MS patients after oxygen-ozone therapy. *miR-17* is an overexpressed miRNA in MS patients associated with Treg cells. It promotes the differentiation of Th1 cells leading to inflammatory responses. *miR-17* is introduced as a negative regulator of inhibitory activity, proliferation, and trafficking of Treg cells. It has also been reported that the expression of *FoxP3* and related cytokines of Treg cell are suppressed by *miR-17*. Accordingly, *miR-17* has a potential role in the progression of MS disease (Yang et al., 2016). *miR-27*, an upregulated miRNA in Treg cells found in MS patients, has an inhibitory effect on the differentiation and responses of Th2 cells and amplification effects on inflammatory responses of Th1 cells, in

autoimmunity. miR-27 leads to impairing the generation and function of both thymic and peripheral Treg cells. Also, miR-27 negatively regulates the expression of *FoxP3* and *IL-10*. Thereby, miR-27 can lead to MS development by suppressing the Treg cells (Cruz et al., 2017). miR-146 is an overexpressed miRNA in Treg cells that mediates the differentiation of them. miR-146a acts as a negative regulator of immune responses and inhibits the expression of inflammatory agents such as *STAT1*, *TRAF6*, and *IRAK1*. In MS patients, an increased expression level of miR-146a has been observed in Treg cells, which is required for their activity. According to previous studies, the increased expression level of miR-146a has been evidenced in active lesions of experimental autoimmune encephalomyelitis and PBMCs of MS patients. These data suggest the potential role of miR-146a in MS pathogenesis (Li et al., 2015). So, regulation of the mentioned miRNAs would augment the activation and function of Treg cells as well as the secretion of TGF- β and IL-10 cytokines, which can cause MS improvement.

Up to now, studies have demonstrated no complete and adequate outcomes on the ground of oxygen-ozone therapy in MS. However, some related studies to MS and other disorders reported the efficient antioxidant and anti-inflammatory activity of oxygen-ozone therapy, which can be considered as a powerful therapeutic regimen in MS patients. It can say, results of the current study confirm the previously reported outcomes in terms of the anti-inflammatory function of oxygen-ozone therapy, which can alleviate the inflammation and contribute to the disease remission. Currently, Izadi et al. (2020) evaluated the O₃-AHT on Th17 cell frequency and its mediated factor in MS patients compared to healthy controls. Their findings illustrated the significant reduction in the number of Th17 cells, the expression levels of *ROR γ t*, *IL-17*, *miR-141*, and *miR-155*, as well as the remarkable decrease in the secretion level of IL-17 cytokine in MS patients after treatment with ozone when compared with pretreatment condition and healthy subjects.

In another study by Lintas et al. (2013), O₃-AHT was investigated in MS patients. Accordingly, near-infrared spectroscopy (NIRS) system was used to monitor the oxygenation in MS cases. Findings revealed the decreased chronic oxidative stress and altered level of cytochrome C oxidase quantity after oxygen-ozone therapy. Also, it has been reported that oxygen-ozone therapy increased cerebral blood flow and activity in MS patients. Molinari et al. (2014) evaluated the ozone effects in 20 RRMS patients compared to 20 controls using the NIRS system. Their results showed that O₃-AHT reduced hemoglobin dioxygen and enhanced the cytochrome C oxidase. In another study conducted by Simonetti et al. (2014), the oxygen-ozone therapy effect was evaluated in RRMS patients. Promising and improvement effects of oxygen-ozone therapy were found on cramps, pain, vein microcirculation, and sleep rhythms of investigated patients. Moreover, Salem et al. (2016) investigated the oxygen-ozone therapy effectiveness on demyelination derived from ethidium bromide lonely or in combination with Cortona. After oxygen-ozone therapy, GSH level and paraoxonase 1 activity were decreased, but p53, TNF- α , IL-1 β , and IFN- γ levels were increased. According to previous studies, oxygen-ozone therapy also

demonstrated encouraging outcomes in other disorders. As an example, Kaya et al. (2017) assessed the oxygen-ozone therapy effect on a rat model of experimental uveitis. Study results evidenced the anti-inflammatory activity of ozone, which can decrease the levels of TNF- α , IFN- γ , and IL-6 cytokines. In another study, Izadi et al. (2019) assessed the oxygen-ozone therapy efficacy on diabetic foot ulcer healing. They reported that oxygen-ozone therapy with systemic and local administration led to wound healing and complete wound closure by reducing the FBS level and antioxidant function. Also, it has been shown that the inflammation was reduced following the significant reduction in levels of C-reactive protein and erythrocyte sedimentation rate with oxygen-ozone therapy.

5 | CONCLUSION

Findings of the current study indicated the effectiveness of oxygen-ozone therapy in treating MS patients. Oxygen-ozone therapy increased the frequency of Treg cells requiring for the maintenance of tolerance and improvement of the autoimmunity. The elevated expression levels of *FoxP3* and TGF- β were also found as beneficial effects of oxygen-ozone therapy, which have a role in the differentiation and development of Treg cells. Considerably, oxygen-ozone therapy decreased the expression level of *miR-17*, a negative regulator of Treg cell, and increased the expression level of *miR-146a*, required for the suppressive function of Treg cell in MS-treated patients. Thereby, oxygen-ozone therapy would be a potent and effective therapeutic approach to decrease inflammation and improve MS patients.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

Data supporting the findings in this study are immediately available upon reasonable request.

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