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Changes in Th17 cells frequency and function after ozone therapy used to treat multiple sclerosis patients

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ABSTRACT

Background: : Multiple sclerosis (MS) is a neurodegenerative autoimmune disease with chronic inflammation. In the course of the disease, the increased levels of Th17 cell, and its relevant inflammatory factors, may cause disease inflammation and progression. Ozone therapy with anti-oxidant and anti-inflammatory functions is known as a beneficial therapeutic approach. The current non-controlled study aimed to evaluate the therapeutic implications of ozone autohemotherapy on Th17 responses in MS patients.

Methods: : 20 MS patients as the experimental group received ozone therapy (100 ml of O2/O3 compound (25 ugs/ml concentration) with 100 ml of autologous blood) twice per week for 6 months. The frequency of Th17 cells, gene expression of the relevant factors (RORɣt, IL-17, IL-23, miR-141, miR-155, and miR-200), as well as the secretion levels of IL-17 and IL-23 cytokines, were compared between the patient and control groups, as well as the group of patients before and after ozone therapy using the flow cytometry, Real-time PCR, and ELISA techniques, respectively.

Results: : Findings indicated the significant decrease in the frequency of Th17 cells ($P = 0.0002$), the expression levels of RORɣt and IL-17 ($P = 0.0001$ and $P = 0.0004$, respectively), as well as miR-141 and miR-155 ($P < 0.0001$ and $P < 0.0001$, respectively) in post-treatment condition with Ozone compared to pre-treatment condition. Also, the significant reduction in the secretion level of IL-17 ($P = 0.043$) was detected in treated patients.

Discussion: : Since increased levels and responses of Th17 cells may have critical roles in MS pathogenesis and inflammation, our findings revealed that ozone autohemotherapy could lower the Th17 responses in peripheral blood of MS patients and can be a beneficial approach in MS treatment.

1. Introduction

Ozone therapy, as a novel therapeutic approach, has been discovered for various purposes and diseases treatment. Ozone gas (O3) is a potent antioxidant agent that was found out in the 19th century. O3 is made up of three oxygen atoms, which is identified as an unstable, inorganic, and water-soluble molecule (Elvis and Ekta, 2011; Zanardi et al., 2016). Due to the mesomeric states of O3, it has a transient interaction with water and itself. The half-life of O3 is 40 days at 20 C temperature. Ozone has a protective effect against UV rays' side effects (Di Paolo et al., 2004). Currently, it has been reported that ozone has therapeutic functions such as inhibitory effects against bacteria, parasites, and fungal infections (Madrigal, 2007). Several preclinical and clinical trial studies have been conducted to evaluate the ozone therapeutic value in various disorders, including acute and chronic diseases, peripheral vascular disease, cardiovascular, neurological, gastrointestinal, orthopedic, and genitourinary disorders (Smith et al., 2017; Romero Valdes et al., 1993).

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Multiple sclerosis (MS) is a common multifaceted demyelinating and chronic autoimmunity of the central nervous system (Compston and Coles, 2008 Oct 25; S. Dolati et al., 2018). MS development is strongly related to genetics and environmental factors. MS is recognized by neurologic deficits, oligodendrocyte loss, axonal transection, inflammatory condition, and infiltration of inflammatory cells (Ghasemi et al., 2017). Several previous Genomic association investigations have evidenced the relationship between immune system responses and MS pathogenesis (Hafler et al., 2007). Autoreactive lymphocytes, particularly T cells and their interaction with the blood-brain barrier (BBB), have a pivotal role in MS pathophysiology (S. Dolati et al., 2018). Placement of macrophages, plasma cells, T- and B cells in the active edge of lesions have been proved in all stages of MS (Prineas and Wright, 1978). Also, the peripheral blood of MS patients contains the increased number of myelin-reactive activated T cells that have a prominent function in MS development by targeting the self-antigens derived from myelin (Pette et al., 1990). In response to intracellular pathogens and tissue, destruction resulted from autoimmune disorders like MS, T cells are polarized to Th1 and Th17 cells (Severson and Hafler, 2009). Th17 cells are distinguished by secreting the pro-inflammatory cytokines, especially IL-17 which its role was evidenced in EAE mice (Harrington et al., 2005). IL-23 is another correlated cytokine with MS pathogenesis, which has a role in the differentiation and development of Th17 cells, along with RORɣt. RORɣt is a transcription factor required for Th17 cell differentiation and function (Annunziato et al., 2007; Ivanov et al., 2006). It has been notified that the frequency and inflammatory responses of Th1 and Th17 cells are elevated in MS patients, particularly in relapsing-remitting (RR) type. Also, overexpression of IL-17, IL-23, TGF-β, IL-6, and RORɣt have been reported in lesions, CSF, and PBMCs of MS patients. Thereby, given the inflammatory role of Th17 cells and their related factors, these cells may also be important in the inflammation and pathogenesis of MS, along with the inflammatory responses of the Th1 cells (Matusevicius et al., 1999; Tzartos et al., 2008). miRNAs, as single-stranded-noncoding RNAs, are the other related factors to MS pathogenesis. miRNAs regulate the gene expression at the posttranscriptional level as well as immune responses and various biological processes. Based on this, miRNAs repress the expression of the protein-coding genes by translational suppression (S. Dolati et al., 2018). Functional dysregulated miRNAs are correlated with autoimmune diseases, cancers, and neurodegenerative disorders (Kanwar et al., 2010; Sonntag, 2010). The presence of dysregulated miRNAs has been indicated in various autoimmune disorders such as MS, rheumatoid arthritis, psoriasis, and systemic lupus erythematosus (Alevizos and Illei, 2010; Furer et al., 2010; Iborra et al., 2012; Pauley et al., 2009). In MS patients, there are different miRNAs related to the pathogenesis of the disease (Keller et al., 2009). miR-141, miR-155, and miR-200 are the Th17 associated miRNAs that are found as prominent components of MS development. Mentioned miRNAs augment Th17 differentiation, and so, Th17 regulation and MS pathogenesis are powerfully relevant to these miRNAs (Chen et al., 2018). In consequence, suppression of miR-141, miR-155, and miR-200 can restrain the differentiation of the Th17 cells and secretion of its cytokines, including IL-17 and IL-23, which are led to the prevention of inflammation. The current study focused on evaluating the alteration of Th17 cells, related cytokines (IL-17 and IL-23), transcription factor (RORɣt), and miRNAs (miR-141, miR-155, and miR-200) before and after ozone therapy in MS patients.

2. Methods

2.1. Study design and patients

This study was piloted in a non-controlled study. All patients who entered the present study received weekly interferon beta-1a (Actovex) (Gemabiotech S A, Argentina) injections for at least 3 months before the intervention, and also they were weekly under Actovex treatment during supplementation. Before the intervention, patients were examined based on neurological damage and the results of brain magnetic resonance imaging (MRI) and laboratory tests. Additionally, we enrolled 20 -matched healthy control group. Written informed consent was taken from all included patients and the healthy control group under a protocol approved by the Ethics Committee of Baqyatallah University of Medical Sciences (IR.BMCU.REC.1398.041). Patients were treated with ozone therapy, and blood samples were gathered before and after treatment. The inclusion criteria of the current study were willingness of study population and the distinction of RRMS patients according to clinical symptoms and MacDonald criteria. Moreover, Bedridden and pregnant patients, over 75 years old population, patients with coagulation and platelet disorders, hypocalcemia, cancers, other autoimmune diseases, blood systemic disorders, thyroid dysfunction, and ozone sensitive individuals were excluded from the study. Demographic information and clinical features treated patients and healthy control groups were presented in Table 1.

Table 1
Sequences of Primers.

Gene	Primer	Sequence
IL-17	Forward	CATAACCGGAATACCAATACCAAT
	Reverse	GGATATCTCTCAGGGTCCTCATT
IL-23	Forward	GGACAACAGTCAGTTCTGCTT
	Reverse	CACAGGGCTATCAGGGAGC
RORγt	Forward	ACTCAAAGCAGGAGCAATGGAA
	Reverse	AGTGGGAGAAGTCAAAGATGGA
β-Actin	Forward	AGAGCTACGAGCTGCCTGAC
	Reverse	AGCACTGTGTTGGCGTACAG
RNU6	Forward	CTCGCTTCGGCAGCACATATACT
	Reverse	ACGCTTCACGAATTTGCGTGTC

2.2. The therapeutic method of ozone autohemotherapy

Ozone administered through the major autohemotherapy method, in which a large amount of blood (between 50 and 100 ml) is collected from the patient, exposed to ozone, and re-infused into the patient. To Ozone autohemotherapy, in the morning, 100 ml whole blood was collected from patients via the median cubital vein and transferred into a sterile flask containing 25 U/ml of heparin as an anticoagulant. O2/O3 mixture was generated using the ozone maker device (HAB Herrmann Apparatebau GmbH) (Germany). Ozonized blood was provided by incubating 100 ml of O2/O3 compound (25 ug/ml concentration) with 100 ml of autologous blood for 3 to 5 min along with the gentle rotating movement. Then, this mixture was filtered using the 0.2 u filter and infused back into the same vein of the patient within 15–20 min. Ozone autohemotherapy was administered twice weekly for 6 months.

2.3. Blood sampling and PBMCs isolation

10 ml of blood samples were collected from the patients (before and after ozone therapy) and control groups. To separate the Peripheral blood mononuclear cells (PBMCs), density-gradient centrifugation was performed by using the 1.077 g/ml standard Ficoll (lymphosep) (Biosera, East Sussex, UK) and then, centrifuged at 450 g for 25 min. After twice washings by phosphate-buffered saline (PBS) (Sigma-Aldrich, Schnelldorf, Germany), PBMCs were cultured in 5 ml medium possessing the 10% heat-inactivated fetal bovine serum (FBS), 200mML-glutamine, and 100 U/ml penicillin along with 10 ng/ml of phorbol myristate acetate (PMA) (eBioscience, San Diego, CA). After 48 h of incubation at 37 °C and 5% CO2, the cultured PBMCs and supernatant were collected to determine the gene expression by Real-time-PCR and secretion level of cytokines by Enzyme-linked immunosorbent assay (ELISA).

2.4. Flow cytometry assessment

Flow cytometry technique was used to evaluate the frequency of Th17 cells for the healthy control group and treated MS patients before and after ozone autohemotherapy. To IL-17 intracellular staining, isolated cells were incubated at 37 °C in a 5% CO2 incubator for 5 h, in the presence of 0.5 μM ionomycin, 10 ng/mL of PMA (Sigma-Aldrich, Schnelldorf, Germany), and monensin (eBioscience, San Diego, CA). Then, after washing, anti-CD4-APC (BD Biosciences, San Jose, CA) was used to incubate with cells for 15 min at 4 °C. After incubation, isolated cells were washed twice and permeabilized by fixation/permeabilization buffer (eBioscience). Moreover, to intracellular staining, cells were incubated with FITC-labeled anti-human IL-17 for 20 min at room temperature. In this investigation, FITC and APC mouse IgG1, κ-isotype control were isotype controls. Consequently, CD4 + cells with secreting IL-17 cytokine were counted as Th17 cells (Abdolmohammadi Vahid et al., 2019; S. Dolati et al., 2018).

2.5. Real-time PCR assay

Expression level of the Th17 transcription factor (RORγt), related cytokines (IL-17 and IL-23), and miRNAs (miR-141, miR-155, and miR-200) were measured by Real-time PCR in cultured PBMCs of the healthy control group and MS patients before and after ozone therapy. Based on this, SYBR green method, along with specific forward and reverse primers, were used to assay. RNX-PLU solution (SinaClon, Tehran, Iran) and Revert Aid Reverse Transcriptase Kit (Thermo Fisher Scientific, Waltham, MA) were used to RNA extraction and complementary DNA synthesis (cDNA), respectively. Then, Real-time PCR was performed according to the following steps to quantify the mRNA gene expression. At first, the denaturation step was initiated and repeated for 40 cycles at 95 °C for 10 s; annealing step was continued at 60 °C for 30 s and followed by extension step for 20 s at 72 °C. 10-fold serial dilutions of genes concentrated samples were used to provide the six standards which were applied to plot the Standard curves. Electrophoresis analysis on 2% agarose gel and DNA sequencing by Biosystems (Seqlab, Gottingen, Germany) were implemented to validate the amplification. For comparing the expression of target genes, the U6 small nuclear (RNU6) housekeeping gene was used for miRNAs and β-actin was used for transcription factor as an endogenous control. Consequently, data analysis was applied by the comparative Ct method using the $2^{-\Delta\Delta Ct}$ formula to analyze the expression relative to the endogenous gene and normalize the expression folds of target genes (Ahmadi et al., 2019). The sequence of primers is listed in Table 2.

2.6. ELISA assay

Secretion levels of Th17 related cytokines (IL-17 and IL-23) were assessed by the enzyme-linked immunosorbent assay (ELISA) kit (MyBioSource, San Diego, CA) in the supernatant of cultured PBMCs in healthy control subjects and MS patients before and after ozone therapy. The summary of the procedure is as follows: 100 μL of coating antibody was applied to coat the ELISA plate overnight. After coating, the plate was washed by PBS containing 0.05% Tween-20 and incubated with a blocking buffer on a shaker for one hour. In the next step, 100 μL of samples and standards were poured into wells; incubated for an hour, and washed. The wells were incubated by 100 μL of a biotinylated-antibody for one hour, which was followed by the addition of 100 μL of the Streptavidin-HRP complex for 30 min and then washed. Afterward, 100 μL of tetramethylbenzidine (TMB) substrate was added to wells and washed after 20 min. In the last step, the reaction was stopped using the H2SO4 stop solution. Consequently, absorbance values of samples were read by Medgenix ELISA reader (BP-800; Biohit, Helsinki, Finland) at 450 nm.

Table 2
Demographic of the 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), y	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), y	4.2 ± 1.5 (2–8)	–	–

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

2.7. Statistical analysis

We conducted a statistical analysis using the SPSS PC Statistics Software (version 19.0; SPSS Inc, Chicago, IL). Results were presented as mean ± SD. The statistical differences of immunologic variables were compared between MS patient groups and healthy subjects by unpaired Student's *t*-test. Also, the paired Student's *t*-test was carried out to compare the alteration of outcomes between pretreatment and posttreatment patients. We applied the Linear-by-linear association χ^2 test to evaluate the linear trend among the immune parameters groups, the response rate of the parameters, and the consequence of treatment. Graphs were drawn using the GraphPad Prism for Windows (version 7.00; GraphPad Software, La Jolla, CA, https:www.graphpad.com). Results were expressed as mean ± SEM. $P < 0.05$ which were presented as statistically significant.

3. Results

3.1. Frequency of Th17 cells in MS patients and control groups

We assessed the number of circulating Th17 cells in peripheral blood by the flow cytometry technique. The flow cytometric data analysis demonstrated the greater proportion of Th17 cells in the peripheral circulation of MS patients (4.592% ± 1.37%) compared to the healthy controls (3.234% ± 1.155) ($P = 0.0017$) (Fig. 1A and B).

3.2. Alterations of Th17 related transcription factor, cytokines, and miRNAs in MS patients compared to control groups

We conducted the Real-time PCR technique to evaluate the expression levels of RORγt and Th17 related cytokines (IL-17 and IL-23). Data analysis indicated that mRNA expression of RORγt was considerably high in MS patients in comparison with the control groups (1.657 ± 0.698 vs 1.005 ± 0.069, $P = 0.0018$) (Fig. 1B). Also, the expression levels of IL-17 and IL-23 cytokines in MS patients were compared to healthy subjects by real-time PCR. As a result, we observed the considerably enhanced expression of IL-17 and IL-23 mRNAs in patients than controls (1.643 ± 0.901 vs 1.002 ± 0.074, $P = 0.0038$ and 1.713 ± 0.708 vs 1.005 ± 0.071, $P < 0.0001$, respectively) (Fig. 1C). Moreover, we quantified the levels of IL-17 and IL-23 inflammatory cytokines of secreted into the culture supernatants of PBMCs by ELISA technique. In comparison, the means of cytokine expression of both IL-17 and IL-23 were in higher level in MS patients than control groups (144.7 ± 53.13 vs 91 ± 44.91, $P = 0.0014$ and 236.2 ± 101.8 vs 155.8 ± 86.08, $P = 0.01$, respectively) (Fig. 1D). We also compared the expression levels of miR-141, miR-155, and miR-200 between patient groups and control groups by Real-time PCR. Findings showed the remarkable enhanced in the expression levels of miRNAs in patients compared to healthy controls (1.803 ± 0.888 vs 0.996 ± 0.094, $P = 0.0004$; 3.678 ± 1.958 vs 1.011 ± 0.083, $P < 0.0001$; and 2.331 ± 1.207 vs 1.014 ± 0.082, $P < 0.0001$, respectively) (Fig.2).

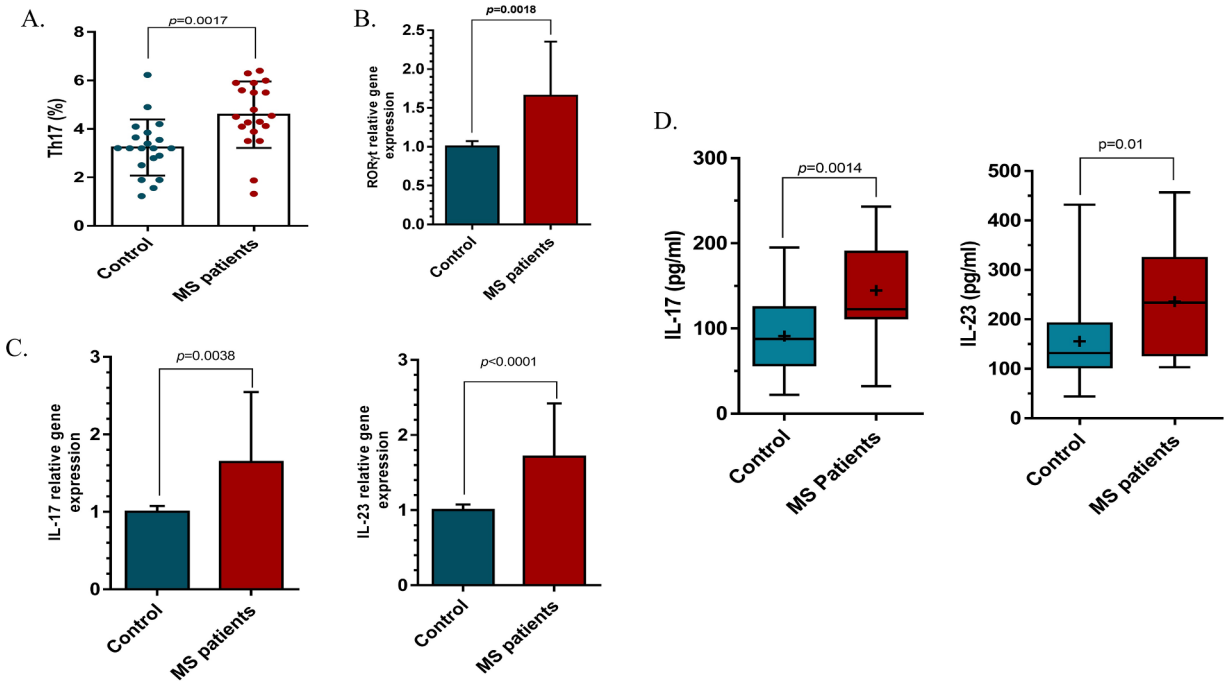

Fig. 1. Alteration of Th17 Cell and its related factors in MS patients compared to control groups. A. According to flow cytometric data analysis, frequency of Th17 cells in MS patients was significantly greater compared to control groups ($P = 0.0017$). B. RORγt expression level was found that significantly higher in MS patients in comparison with controls ($P = 0.0018$). C. Expression levels of both IL-17 and IL-23 were detected that notably increased in MS patients than healthy subjects ($P = 0.0038$ and $P < 0.0001$, respectively). D. Significant raise was observed in secretion levels of both IL-17 and IL-23 in MS patients when compared with control groups ($P = 0.0014$ and $P = 0.01$, respectively). MS patient group, $n = 20$; control group, $n = 20$. Results were presented as mean ± SD. $P < 0.05$ was described as statistically significant. MS, multiple sclerosis; Th17, T-helper 17; RORγt, RAR-related orphan receptor γ; IL, interleukin.

3.3. Th17 frequency before and after ozone therapy

After ozone therapy, we measured the frequency of circulating Th17 cells in the peripheral blood of MS patients before and after treatment by the flow cytometry technique. Our flow cytometric results illustrated that the level of Th17 cells was markedly lessened from 4.308% ± 1.411% in post-treatment patients to 3.500% ± 1.163% in patients before treatment ($P = 0.0008$) (Fig. 3A and B).

3.4. Alterations of RORγt, IL-17, IL-23 and miRNAs in ms patients in pre- and post-treatment condition with Ozone

We quantified the expression levels of RORγt, IL-17, IL-23, miR-141, miR-155, and miR-200 using the Real-time PCR in MS patients before and after ozone autohemotherapy. We found the significantly decreased level of RORγt expression was after treatment with ozone compared to the patients before treatment (0.635 ± 0.253 vs 0.992 ± 0.086, $P = 0.0001$) (Fig. 3C). Also, we examined the expression levels of IL-17 and IL-23 cytokines in MS patients. Significantly, ozone therapy reduced the expression levels of IL-17 in patients after treatment (0.639 ± 0.361 vs 1.003 ± 0.074, $P = 0.0004$).

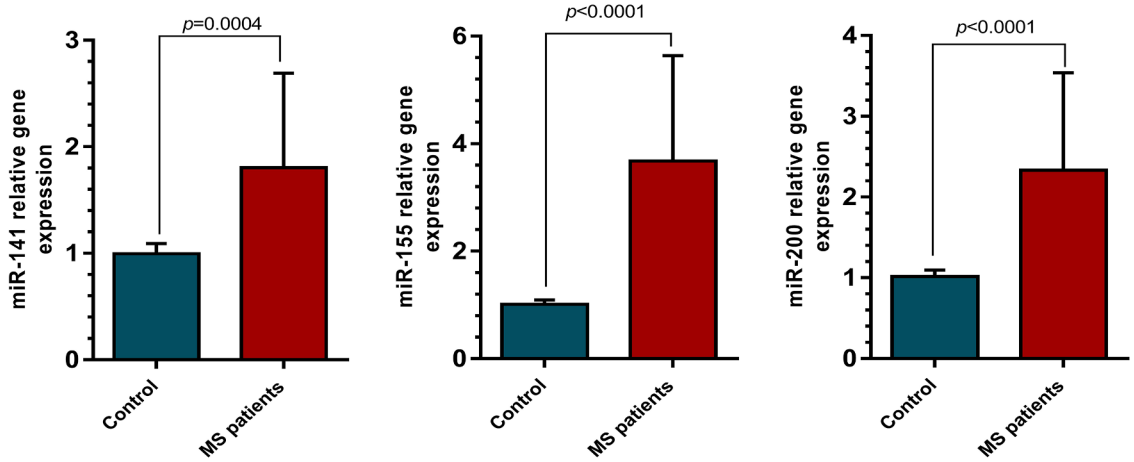

Fig. 2. MicroRNAs expression levels in MS patients compared to controls. Th17 related miRNAs (miR-141, miR-155, and miR-200) demonstrated the considerable higher expression levels in MS patients when compared to healthy subjects ($P = 0.0004$, $P < 0.0001$, and $P < 0.0001$, respectively). MS patient group, $n = 20$; control group, $n = 20$. Results were presented as mean ± SD. $P < 0.05$ was described as statistically significant. MS, multiple sclerosis; Th17, T-helper 17; miRNA, microRNA.

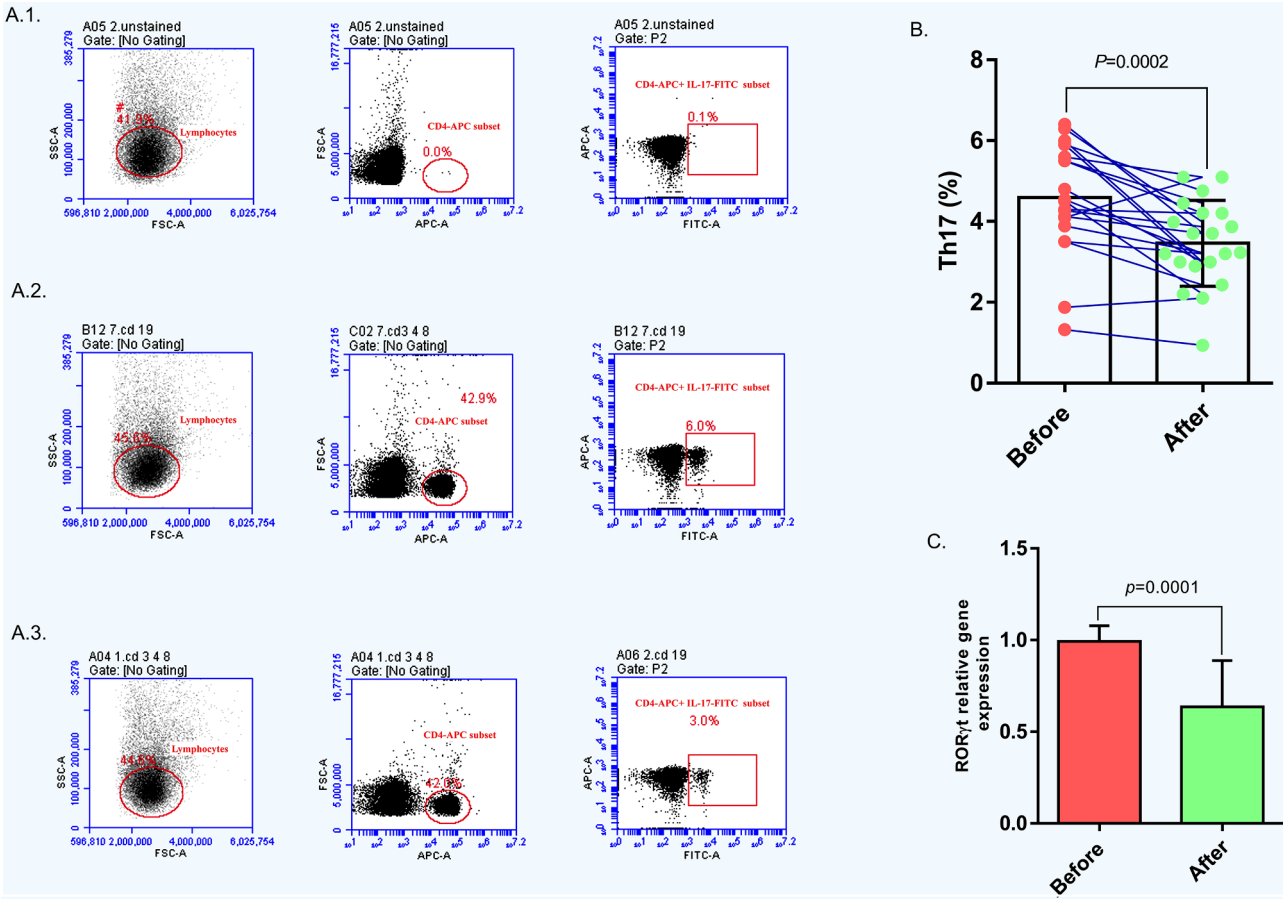

Fig. 3. Frequency of Th17 cells and expression level of RORγt in MS patients before and after Ozone autohemotherapy. A. Th17 cells gating was firstly performed based upon side scatter and CD4-APC and secondly based upon IL-17-FITC and CD4-APC. A.1. The isotype control for the flow cytometric strategy of CD4 + IL-17 + cells, A.2. representative image of a sample with high levels of IL-17 + CD4 + *T* cells, A.3. other samples with a reduced frequency is shown. B. In a comparison of pre- and post-treatment frequency of Th17 cells, a significantly lowered number of Th17 cells was quantified in treated patients with ozone ($P = 0.0002$). C. Treatment with ozone considerably decreased the expression level of RORγt in treated patients in comparison with patients before treatment ($P = 0.0001$). Ozone treated group, $n = 20$. Results were presented as mean ± SD. $P < 0.05$ was described as statistically significant. MS, multiple sclerosis; FITC, fluorescein isothiocyanate; APC, Allophycocyanin; Th17, T-helper 17; RORγt, RAR-related orphan receptor γ.

However, we found no significant decrease in the expression level of IL-23 in treated patients (0.8 ± 0.50 vs 1.019 ± 0.078, $P = 0.091$) (Fig. 4A). The secretion levels of IL-17 and IL-23 were determined by ELISA. After ozone therapy, mean levels of IL-17 and IL-23 were notably decreased from 144.7 ± 53.13 pg/ml and 236.2 ± 101.8 pg/ml to 116.9 ± 53.83 pg/ml and 173.8 ± 78.56 pg/ml, respectively. As a result, a significant reduction in the levels of IL-17 was obtained in post-treatment patients when compared to pre-treatment ($P = 0.043$); however, treated patients showed no significant drop in secretion level of IL-23 ($P = 0.07$) (Fig. 4B). Besides, we compared the expression levels of miR-141, miR-155, and miR-200 in patients before and after treatment. After ozone therapy, expression levels of miRNAs were assessed and found that the levels of miR-141 and miR-155 were significantly lowered in treated patients than the patients before treatment (0.638 ± 0.291 vs 1.019 ± 0.078, $P < 0.0001$ and 0.317 ± 0.202 vs 1.019 ± 0.078, $P < 0.0001$, respectively). Also, no significant reduction was detected in the expression level of miR-200 between pre-treatment and post-treatment patients (0.845 ± 0.378 vs 1.019 ± 0.078, $P = 0.071$) (Fig. 5).

3.5. The total EDSS before and after ozone therapy in MS patients

After ozone therapy, the EDSS in patients significantly decreased from 1.18 ± 0.39 to 0.83 ± 0.28 ($P = 0.032$) compared with before treatment.

4. Discussion

MS is a chronic inflammatory disease distinguished by the myelin destruction of the central nervous system and classified as an autoimmune disease. In addition to genetic factors, viruses, metabolism, and environmental agents are known as related risk factors to MS pathogenesis triggering the autoimmune responses (Ghasemi et al., 2017; Hafler, 2004). The myelin sheath destruction and CNS lesions are mostly elicited by the inflammation derived from the focal infiltrated T-lymphocytes and macrophages (Traugott et al., 1983). Although FDA approved drugs have been developed to improve the MS, complete practical therapeutic approaches do not exist for MS. Encouragingly, the remission potential of ozone therapy has been investigated in different diseases. Ozone leads to the initiation of the endogenous cascade and secretion of various biological substrates after inducing oxidative stress (Smith et al., 2017; Bocci et al., 2005). Ozone can dissolve in plasma and generate reactive oxygen species (ROS) products by reacting with water and polyunsaturated fatty acids (PUFA) and Lipid ozonation products (LOP) (Inal et al., 2011).

Besides, Ozone therapy can regulate the immune system responses and keep its balance through triggering or suppressing the immune system based on the type and condition of diseases. Briefly, Ozone therapy possesses several advantages as follows: 1) Improvement of oxygen-carrying capability of RBCs, the glycolytic pathway, and oxygen delivery, 2) Upregulation of intracellular enzymes with antioxidant

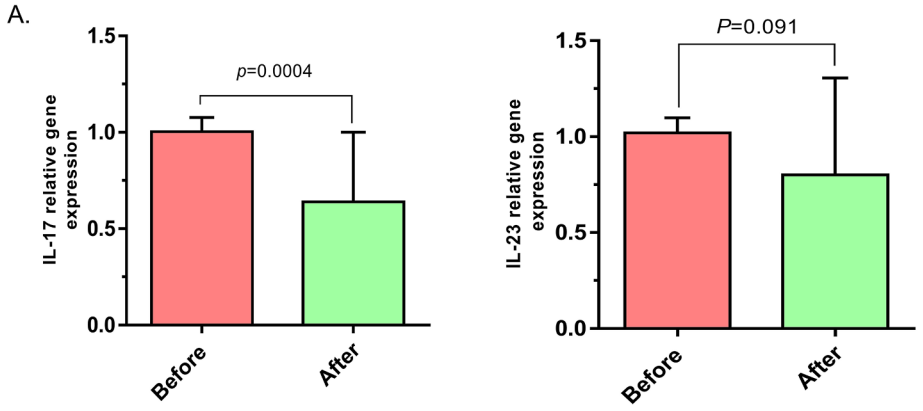

Fig. 4. The expression and secretion levels of IL-17 and IL-23 in MS patients pre- and post-treatment with ozone. A. After ozone autohemotherapy, a significant reduction in the expression levels of IL-17 and non-significant difference in the expression level of IL-23 were observed in treated patients ($P = 0.0004$ and $P = 0.091$, respectively). B. The secretion level of IL-17 was remarkably diminished in treated patients with ozone ($P = 0.0004$). However, the non-significant difference of the IL-23 secretion level was observed between pre- and post-treatment patients ($P = 0.091$). Ozone treated group, $n = 20$. Results were presented as mean ± SD. $P < 0.05$ was described as statistically significant. MS, multiple sclerosis; Th17, T-helper 17; IL, interleukin.

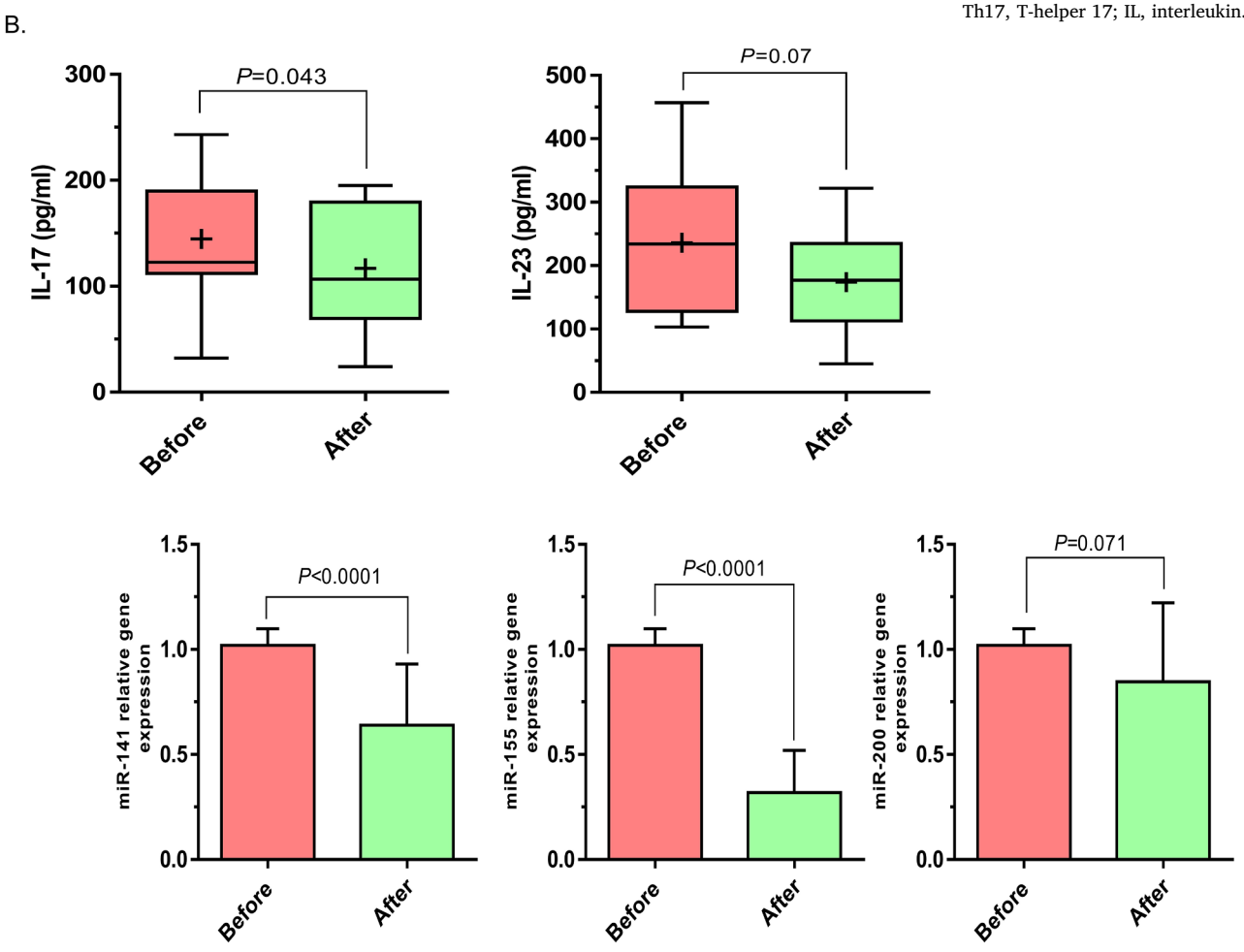

Fig. 5. Expression levels of Th17 related miRNAs in MS patients before and after Ozone therapy. Treatment with ozone significantly reduced the expression levels of miR-141 and miR-155 in treated patients ($P < 0.0001$ and $P < 0.0001$, respectively); however, no significant reduction was found in the expression level of miR-200 in MS patients after ozone therapy ($P = 0.071$). Ozone treated group, $n = 20$. Results were presented as mean ± SD. $P < 0.05$ was described as statistically significant. MS, multiple sclerosis; Th17, T-helper 17; miRNA, microRNA.

function, 3) Induction of immune system responses and secretion of cytokines by producing the hydrogen peroxide (H2O2), 4) Alleviation of patients' pain. Thereby, these mentioned clinical benefits may result in the proper treatment of MS patients (Bocci, 1994; Bocci, 2005; Hu et al., 2018). However, the effectiveness of ozone therapy on the immune system and its regulatory capability on maintaining the immune system balance are not clarified. Considering that Th17 cell responses as inflammatory mediators may also play an inflammatory role in MS pathogenesis, ozone therapy would be advantageous in improving the disease by affecting Th17 cell responses. In the current study, we investigated the ozone autohemotherapy implications on the frequency of Th17 cells and alteration of associated factors, including transcription factor (RORγt), cytokines (IL-17 and IL-23), and miRNAs (miR-141, miR-155, and miR-200) in 20 MS patients before and after treatment. Further, the alterations of the mentioned factors were compared between the patient group and healthy controls before the intervention. The flow cytometric analysis results demonstrated that the levels of Th17 cells were significantly declined after ozone therapy compared to non-treatment patients. According to the previous studies, the initiation and development of MS are strongly related to T cells. Considerably, Th1 and Th17 elicit the MS inflammation and progression by producing the inflammatory cytokines (Severson and

Hafler, 2009). Th17 is implicated in several autoimmune diseases and introduced by secreting the IL-17A to IL-17F, IL-21, IL-22, and IL-26 pro-inflammatory cytokines (Harrington et al., 2005). Moreover, we examined the mRNA expression of RAR-related orphan receptor gamma t (RORγt), a transcription factor of Th17, using the Real-time PCR method before and after ozone therapy. Data analysis has shown that the gene expression of RORγt prominently reduced in treated patients with ozone when compared to the patients before ozone therapy. RORγt differentiates the naive CD4+ *T* cells to Th17 cells and boosts the IL-17 production (Annunziato et al., 2007). Herein, we also assessed the efficacy of ozone therapy on the expression levels of IL-17 and IL-23 cytokines by Real-time PCR. As a result, ozone autohemotherapy meaningfully decreased the mRNA expression levels of IL-17 in treated patients compared to untreated ones; however, we obtained no significant lower in the expression level of IL-23. Besides, we detected the secretion levels of IL-17 and IL-23 cytokines by ELISA. We found the significant and non-significant reduction in IL-17 and IL-23 levels, respectively, in patients after ozone therapy compared to patients before treatment. IL-23 is termed as a heterodimeric cytokine correlating with autoimmune inflammations. IL-23 required for Th17 induction and expansion. It acts as a stabilizer factor of RORɣt leading to the affective responses of Th17 cells (Langrish et al., 2005). Brain local inflammation may be originated from the modulatory function of IL-23 mediating the interaction between T cell and microglia (Li et al., 2007). IL-17 is another important inflammatory cytokine produced by Th17, which has a momentous role in MS pathogenesis. The greater level of IL-17 is associated with the number and severity of active lesions of the brain (Tzartos et al., 2008). It has been evidenced that IL-17, elicits the BBB disruption and disease progression through the producing reactive oxygen species. Based on previously published results, an increased level of Th17 cells, as well as higher mRNA expression and secretion levels of IL-17 and IL-23 cytokines have been reported in acute and chronic active lesions of brain tissue, peripheral blood and cerebrospinal fluid (CSF) of MS patients (Tzartos et al., 2008; Ishizu et al., 2005). Effectively, Th17 disrupts the BBB and causes neurological damage by releasing the cytolytic granzyme B, IL-17, IL-22, CXCL1, CXCL2, and more other inflammatory factors. Hence, the accessibility of Th17 cells to brain tissue is led to the recruitment of other T CD4+ cells from the systemic circulation into CNS (Tzartos et al., 2008; Kebir et al., 2007). miRNAs are the other effective factors associating with MS pathogenesis. miRNAs are non-coding RNAs with the regulatory role in mRNA gene expression. Transcription factors initially control the miRNA expression at the transcription level. miRNAs have prominence roles in cellular development and other biological function such as inflammation, oncogenesis, metabolism, and apoptosis. Immunologically, miRNAs have a substantial role in regulating innate and adaptive immune responses. miRNAs adjust the differentiation, proliferation, survival, and death of B- and T cells (S. Dolati et al., 2018; O'connell et al., 2010). Various autoimmune diseases, including MS, Sj¨ogren's syndrome, systemic lupus erythematosus, psoriasis, and rheumatoid arthritis, are prominently correlated with dysregulated miRNAs. Managing the expression level of miRNA determines the prevention or progression of cancers and autoimmune diseases (Pauley et al., 2009; Jeker and Bluestone, 2010). Based on this, we measured the expression levels of miR-141, miR-155, and miR-200 by real-time PCR. Data analysis unveiled the significant diminishing in expression levels of miR-141 and miR-155 miRNAs and a nonsignificant difference in the expression level of miR-200 after ozone therapy. miR-141, miR-155, and miR-200 are correlated miRNAs with Th17, which have a role in MS pathogenesis. The high expression levels of them have been reported in active lesions of MS patients (Tufekci et al., 2011). Dysregulated miR-141 and miR-200 miRNAs have an essential role in various autoimmune diseases such as inflammatory bowel disease (IBD), psoriasis, ankylosing spondylitis, lupus erythematosus (SLE) and multiple sclerosis. The up-regulation of miR-141 and miR-200 causing the MS development has been reported in MS patients (Naghavian et al., 2015).

Both miR-141 and miR-200 elicit the differentiation of Th17 cells, augment their production and proliferation, and avoid the differentiation of Tregs by suppressing the SMAD2, GATA3, and FOXO3 genes. Potentially, this mentioned miRNAs increase the frequency of Th17 cells and promote the production of IL-17 and IL-22 cytokines (Naghavian et al., 2015). miR-155, as a proinflammatory factor, is one of the most involved miRNAs in autoimmune diseases. miR-155 is overexpressed in MS disease and links to the disease pathogenesis. The enhanced level of miR-155 has been reported in brain tissue lesions and plasma of MS patients. This miRNA differentiates the naive CD4+ *T* cells to Th1 and Th17 cells and triggers the secretion of IFN and IL-17 cytokines. Also, the upregulation of miR-155 induces the production of IL-6 and IL-23 required for Th17 differentiation (Zhang et al., 2014). Overall, mir-155 progresses the disease through elevating inflammation in MS patients.

In consequence, inhibition of mentioned miRNAs can result in suppressing the Th17 responses and the inflammation of MS, as well. From a long time ago, ozone therapy has indicated the successful outcomes in treating various diseases. The anti-oxidant- and the anti-inflammatory effects of ozone therapy have been investigated in different inflammatory and autoimmune diseases such as lung injury, uterine adhesions, bladder toxicity, pyelonephritis, colitis, pancreatitis, dentistry disorders, wound healing, rheumatoid arthritis, and multiple sclerosis (Chen et al., 2013; Kaldirim et al., 2014; Lintas et al., 2013; Uysal et al., 2010). Altogether, our findings are in agreement with reported results from previous investigations on anti-oxidant and anti-inflammatory features of ozone therapy. According to different studies, ozone can decrease oxidative stress and inflammation condition which can lead to alleviation of disease.

In a study, SALEM et al. (Salem et al., 2016) investigated the combination effect of ozone therapy and corton on demyelination derived from ethidium bromide in Rat models of MS. Results indicated the elevated level of p53, IL-1β, TNF-α, and IFN-y and decreased level of paraoxonase 1 functionality and GSH after ozone therapy. In another investigation, Molinari et al. (Molinari et al., 2014) revealed the increased level of cytochrome oxidase C after ozone therapy in MS cases. Moreover, Lintas et al. (Lintas et al., 2013) investigated the ozone autohemotherapy effects on MS patients, which demonstrated the altered level of cytochrome oxidase C and reduced levels of chronic oxidative stress. In a study conducted by Simonetti et al. (Simonetti et al., 2014), improving outcomes have been reported from 23 relapses remitting MS cases and 11 primary/secondary cases that were treated by ozone. Currently, some other studies support our findings in terms of the ozone therapy usefulness in treating neurological disorders and inflammatory and degenerative neurological diseases, like MS (Javad et al., 2020; El-Mehi and Faried, 2020; Di Mauro et al., 2019). Other more studies have also investigated the ozone therapeutic effects on other disorders. Ozone therapy leads to improve the daily function and symptoms of Parkinson patients (Morelli et al., 2018). In a study by Izadi et al. (Izadi et al., 2019), it has been shown that ozone therapy could improve the diabetic foot ulcer healing and lead to wound closure through the antioxidant activity and reduction of FBS, ESR, and CRP levels.

In conclusion, the current study reported the considerable reduction in the frequency of Th17 cell and its associated transcription factor (RORɣt), cytokines (IL-17 and IL-23), and miRNAs (miR-141, miR-155, and miR-200), which result in efficient protection against MS inflammation using the ozone therapy. Hopefully, ozone therapy due to the possessing anti-oxidant and anti-inflammatory activities may contribute to MS treatment by reducing the oxidative stress and inflammation and maintaining the self-tolerance in MS patients.

Author statement

All authors have been involved in drafting the manuscript. The author M. A and M.E designed the project and critically reviewed the

manuscript content.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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