SHORT PAPER

The Effect of Plasma Exchange on the Expression of FOXP3 and RORC2 in Relapsed Multiple Sclerosis Patients

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ABSTRACT

Background: Lack of sufficient information on the mechanism of plasma exchange (PE) therapy in multiple sclerosis (MS), has limited this treatment to individual patients with severe relapses who have been refractory to other treatments. This is while PE is used very successfully as a first-line standard treatment in many other neuro-immune disorders. Recent data suggest that Treg/Th17 counterbalance may indicate the boundaries between promotion and regulation of inflammatory responses in MS and Treg/Th17 ratio may be useful as a marker for monitoring the efficiency of MS therapies. **Objective:** To evaluate the effect of PE on the frequency and ratio of Treg/Th17 cells through concomitant measurement of the expression levels of Treg and Th17 lineage specific transcription factors, FOXP3 and RORC2, respectively. **Methods:** Peripheral blood mononuclear cells of 8 relapsed MS patients were obtained before and after a complete course of PE therapy and the FOXP3 and RORC2 mRNA levels were assayed using real-time PCR approach. **Results:** No significant change in the expression levels of individual transcription factors existed, but a significant increase in FOXP3/RORC2 ratio (p=0.036) was observed. **Conclusions:** Our results suggest that PE therapy influences Treg/Th17 ratio and this maybe a mechanism by which this procedure exerts its improving effects in MS disease.

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Keywords: Multiple Sclerosis, Plasma Exchange, Th17, Treg

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INTRODUCTION

Multiple sclerosis (MS) is the most common chronic neuro-immune disease in which the orchestrated attack of innate and adaptive immune system against the myelin sheet enveloping axons in the CNS destroys myelin and disrupts the signal transduction through the axons (1).

Plasma exchange therapy (PET) is currently used as an individual decision for treatment of MS patients with severe relapses who do not respond properly to corticosteroids (CS). However, due to the lack of evidences and appropriate studies to recognize mechanism of its action in MS patients, it is not carried out as a long-term disease modifying strategy in remission phase. This is while PE is established as a standard therapy for similar neuro-immune disorders, like Guillain-Barré syndrome, chronic demyelinating polyneuropathy, myasthenia gravis, and Lambert–Eaton syndrome (2,3).

Tregs and Th17 cells have been highlighted in the recent studies as the most important controllers and promoters of MS disease, respectively. Since Treg and Th17 cells are related and Tregs can be reprogrammed to become Th17 cells in the presence of TGF-β and IL-6 (4), Treg/Th17 counterbalance may indicate the boundaries between promotion and regulation of inflammatory responses in auto-immune conditions like MS. Also this ratio is an indicator for approximating regulatory and effector functions of these subsets beside their frequency. Therefore,Treg/Th17 ratio can be used as the most helpful marker for evaluating and comparing the effectiveness of different therapies of MS disease.

The present study was conducted to evaluate the effect of PE therapy on the frequency and ratio of Treg/Th17 cells through concomitant measurement of the expression levels of their lineage-specific transcription factors (FOXP3 for Tregs and RORC2 for Th17 cells) in the peripheral blood mononuclear cells of patients before and after treatment.

MATERIALS AND METHODS

Patients. Peripheral blood samples were obtained from MS patients attending a MS outpatient clinic at Kashani University Hospital in Isfahan, Iran. According to McDonald diagnostic criteria for MS, the involved patients were diagnosed as having RR-MS, with a clinically documented attack of neurological symptoms (relapse) not responding to corticosteroid pulse therapy. Relapse was defined as the appearance of one or more neurological aberrations without the presence of any underlying infectious disease. The abnormalities had to persist for more than 24 hours after a period of at least 30 days of disease stability to be considered as a new relapse. All patients had white matter lesions on their brain and/or spinal cord identified via MRI. Clinical severity of disease was measured by the Kurtzke's Expanded Disability Status Scale (EDSS) scores. All patients had been previously treated with interferon-βa during last remission; however patients who had received drug in the last 5 days were not included in this study. The patients, whose severe symptoms of the last relapse had not been relieved by corticosteroid pulse therapy, were indicated to receive the alternative treatment, PE therapy. Eight patients underwent a complete course of PE therapy (5 day, exchanging 1-1.5 plasma volume per day), and their peripheral blood samples were collected immediately before and after the treatment. Informed consents were obtained from all subjects. The study was approved by institutional review boards of Isfahan University

of Medical Sciences and the local ethics committee. More clinical and demographic data of patients has been summarized in Table 1.

Table 1.Demographic data of patients.

Age ± SD, Year (Range)	35.88 ± 9.49 (24-50)
Gender	8 Female/0 Male
Disease duration, Month (Range)	83.25 ± 76.73 (12-252)
EDSS score ± SD (Range)	3.44 ± 1.72 (2.5-6)
	8

Plasma Exchange Therapy. Plasma exchange was carried out using an automated blood cell separator (PCS2 Plasma Collection System, Haemonetics, USA). After the insertion of a femoral or jugular line to allow adequate blood flow. 30-40 mL/kg of plasma (1-1.5 plasma volumes) was removed at each procedure and replaced with isotonic 5.0% human albumin solution.

The following formula (5) was used to estimate the plasma volume to be exchanged for each patient and 1 to 1.5 plasma volume exchanged per procedure.

Estimated plasma volume (in liters) = 0.07 x weight (kg) x (1 - hematocrit).

The procedure was repeated 5 days for each patient. The blood samples were collected before initiating the first-day procedure and after the end of the fifth-day procedure.

RNA Extraction and Real-Time PCR. Peripheral blood mononuclear cells (PBMCs) were isolated using ficoll density gradient method within two hours after sampling and were stored in 300 µl RNA protect Cell Reagent (Qiagen) in -80°C until the next steps. Total RNA was extracted by Trizol reagent (invitrogen) according to the manufacturer protocol. One microgram of total RNA was used to generate single-stranded cDNA using RevertAid^{TM} First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer protocol.

The real-time polymerase chain reaction was performed using SYBRGreen/RoxqPCR Mastermix (Thermo Fisher Scientific) and the StepOne Plus™ quantitative Real time PCR detection System (Applied Biosystems). The reactions conducted in 20 µl, with 10 µl SYBR® Green, 200 µM forward and reverse primers and 1.5 µl cDNA. The following PCR cycling program was used: primary denaturation in 95° for 10 min, denaturation in 95° for 15 sec, annealing and extension in 60° for 1 min for 40 cycles and finally melt curve (increment 0.3 °C, 60°C→95°C) analysis. All experiments were performed in triplicate for each sample. The gene of interest was normalized against the reference gene β actin. The expression level of each target gene was calculated as ΔΔCt method, as previously described. The sequence of specific primers for Real-Time PCR indicated in Table 2 (6).

Statistical Analysis. The relative expression levels of FOXP3 and RORC2 genes were calculated as [2 ΔΔCt], where ΔCt = [Ct (sample) – Ct (β actin gene)] and ΔΔ Ct = [Δ Ct (sample) - Δ Ct (calibrator)]. Statistical analysis was done using SPSS software version

20. The non-parametric tests, Wilcoxon and Freedman, were used and p values‹ 0.05 were reported significant.

Table 2. The sequence of specific primers for Real-Time PCR (6).

primer	sequence
FOXP3-F	5′-GAAACAGCACATTCCCAGAGTTC-3′
FOXP3-R	5′-ATGGCCCAGCGGATGAG-3′
RORC2-F	5′-CAGTCATGAGAACACAAATTGAAGTG-3′
RORC2-R	5′-CAGGTGATAACCCCGTAGTGGAT-3′
ACTB-F	5′-AGCACAGAGCCTCGCCTTT-3′
ACTB-R	5′-GTTGTCGACGACGAGCG-3′

RESULTS and DISCUSSION

Current knowledge about new subsets of pathogenic (Th17 cells) and protective (Tregs) immune cells has made the new medical interventions in MS patients possible. The present study was conducted to study the effects of therapeutic PE on the development and differentiation of these two important cellular players in the disease establishment and promotion. For this purpose we assayed the mRNA expression levels of lineage-specific transcription factors, FOXP3 and RORC2, respectively.

Eight female patients with severe relapses of MS symptoms who did not respond to the routine first line corticosteroid pulse therapy (for three to five days 1 g per day) were indicated to undergo PE therapy instead of a repetition of the pulse therapy (for an additional five days with 2 g per day). This treatment is used routinely for other non-responding MS relapses.

Analyzing the acquired data from quantitative Real time-PCR of PBMCs of patients before and after treatment showed no significant changes in the expression of FOXP3and RORC2 transcription factor (p=0.48 and p=0.20, respectively) (Figure 1). However, when the fold-changes in the expression levels of the two transcription factors were considered as a ratio, FOXP3/RORC2, the relative changes after treatment was significantly positive (p=0.036), meaning that significant increases were occurred in FOXP3/RORC2 ratio with PE treatment (Figure 2).

While PE is increasingly considered as an extraordinary treatment choice in severe relapses of MS which do not respond to CS, it is not carried out as an enduring disease-modifying treatment in MS patients. This is due to the lack of proper studies and the unknown mechanism of action (7). The PE procedure depletes the blood from various immunological factors, such as auto-antibodies, cytokines, chemokines, complement components, and other inflammatory mediators (7). However, previous studies have also confirmed that PE, besides depleting soluble factors from the blood, affects the

frequency, phenotype, function, and cytokine production of different sub-populations of immune cells (8-20).

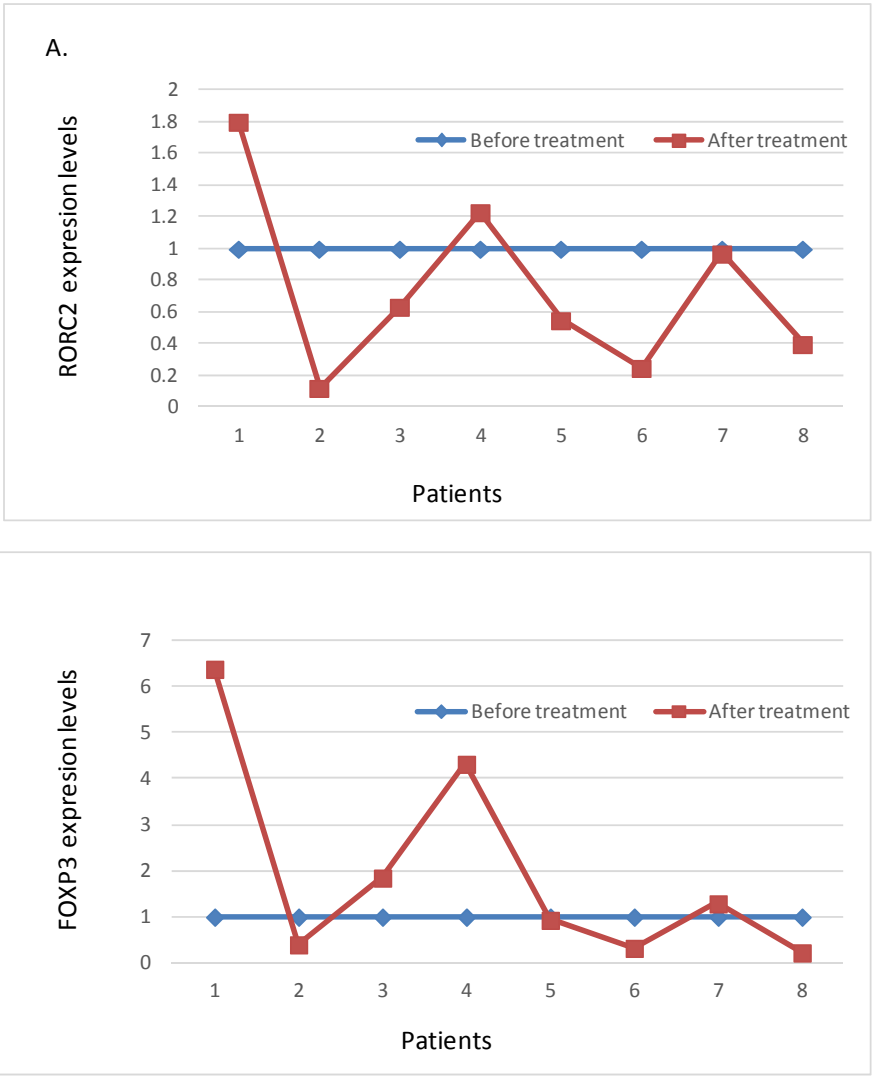
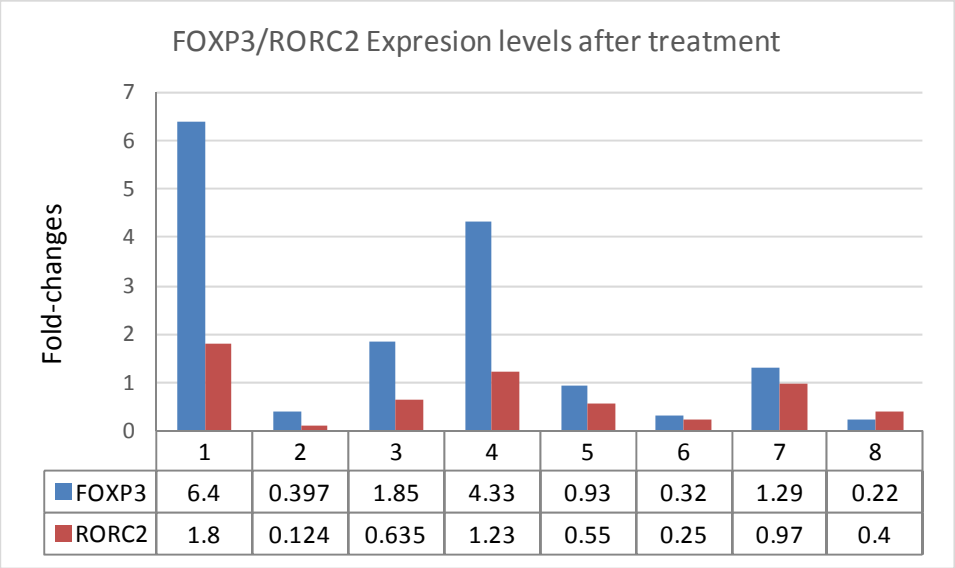


Figure 1. Relative expression levels of FOXP3 (A) and RORC2 (B) transcription factor genes in relapsed MS patients before and after plasma exchange treatment.

Regulatory T cells have been accepted to play the most important role in keeping homeostasis of the immune system and tolerance to self (21,22), and are known as the main regulators of MS immune-pathogenesis (23-25). Th17 is another CD4+ T cell subset which is suspected to promote inflammation in MS, in the absence of efficient control by regulatory cells (26). Accordingly, it seems rational to suppose that influencing the incidence, proportion and function of the mentioned CD4+ T cell pro-inflammatory and regulatory subsets may be one mechanism through which PE exerts its improving effects on MS as a CD4+ T cell-mediated disease. Moreover, in peripheral organs of the immune system, inducible Tregs and Th17 cells are originated from a

common precursor, naive $CD4^+$ T cells. Interestingly, *in vitro* studies have shown that differentiated Treg cells are reprogrammed and converted to Th17 subset, in some definite inflammatory conditions (4).



	1	2	3	4	5	6	7	8
FOXP3	6.4	0.397	1.85	4.33	0.93	0.32	1.29	0.22
RORC2	1.8	0.124	0.635	1.23	0.55	0.25	0.97	0.4

Figure 2. Comparison of fold-changes in the expression levels of FOXP3 and RORC2 transcription factor genes after plasma exchanging.

The opposing immune functions of Treg and Th17 lymphocytes despite their common developmental pathway, alongside Treg-Th17 plasticity, are all suggestive that induced changes in these subsets may not be independent. Regulatory cells suppress effecter Th17 cells, whereas Th17 cells by providing an inflammatory cytokine milieu, may promote conversion of some Tregs to Th17 phenotype, in addition to *de novo* induction of more Th17 cells (4). Therefore, the counterbalance of two subsets defined by Treg/Th17 ratio, which in our study was estimated by FOXP3/RORC2 mRNA expression, can be of more relevance than the prevalence of each subset alone. In this respect, despite not having a significant effect on the FOXP3 and RORC2 mRNA expression levels, the increasing influence of PE on Treg/Th17 ratio which was shown by increased FOXP3/RORC2 mRNA ratio after procedure ($p= 0.036$), can be remarked as a mechanism by which this treatment controls inflammation. Such results may be explained by some immunological facts about inflammation, including: 1-inflammatory milieu can promote differentiation and expansion of Th17 cells (26), 2-inflammatory signals obstacle the induction of regulatory T cells in the periphery (27), and 3-inflammatory conditions may cause differentiated Treg cells to be reprogrammed and converted to Th17 subset, as mentioned above (4). Therefore, diluting the inflammatory cytokines in the peripheral blood trough the plasma exchanging procedure, may overturn all these inflammation effects and cause higher relative quantity of Treg to Th17 which in our results have been shown by higher FOXP3/RORC2 ratio after the procedure.

Looking at the many limitations of our study, including very low number of involved patients, the very short time interval between the last treatment and the blood sampling, we strongly suggest this hypothesis to be examined in other experimental designs, with more patients involved, and at several time points with longer intervals after therapy.

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