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A Randomized, Triple-blind Placebo-controlled Trial to Determine the Effect of Saffron on the Serum Levels of MMP-9 and TIMP-1 in Patients with Multiple Sclerosis

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ABSTRACT

Matrix metalloproteinases-(MMP)-9 facilitates the migration of T-cells to central nervous system (CNS), while (Tissue inhibitor of metalloproteinases-1) TIMP-1 inhibits the function of MMP-9. This study aimed to determine the appropriate treatment option for multiple sclerosis (MS).

Forty-three relapsing-remitting MS (RRMS) patients were randomly divided into two groups of 22 (group A, placebo) and 21 (group B, Saffron pill) individuals. Serum samples were collected from patients' blood before using the Saffron pills/placebo pills and then after 12 months. The serum level of MMP-9 and its inhibitor, as well as TIMP-1, were measured by ELISA kits. Data were analysed using SPSS version 13.

MMP-9 serum levels noticeably decreased in patients with MS following 12 months of treatment with Saffron pills ($p=0.006$) while the changes were not significant before and after 12 months of treatment with placebo pills. Although the levels of TIMP-1 increased significantly after one year treating with Saffron pills ($p=0.0002$), a considerable difference was not observed before and after taking the placebo pills.

The study finding revealed that 12-months treatment with Saffron could have a significant role in reducing the serum level of MMP-9 and increasing the serum level of TIMP-1 in RRMS patients. Therefore, modulating the serum levels of MMP-9 as an important regulator of T cell trafficking to the CNS might be a promising strategy in the treatment of MS patients.

Keywords: Matrix metalloproteinase 9; Multiple sclerosis; Saffron; Tissue inhibitor of metalloproteinase-1

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INTRODUCTION

Multiple sclerosis (MS) is a progressive autoimmune disorder that affects the central nervous system (CNS). Pathologically, it is characterized by demyelination in the spinal cord and brain as well as the presence of inflammatory lesions. The current belief is that MS is an autoimmune disease; characterized by autoreactive T lymphocytes that are originated from the peripheral immune system and migrate to the CNS. Clinically, patients with MS present blurred vision, muscle weakness, fatigue, dizziness, as well as balance, and gait problems. Importantly, current therapies for MS target the inflammatory response, thus highlighting the relevance of further investigation on the immune response in MS.^{1,2} In the United States, alone, there are 400,000 patients^{1,2} with MS and about 2 million patients worldwide. An important area of research in the field of MS is the identification of suitable biomarkers to predict who is at risk of developing MS, biomarkers of disease progression or exacerbation, as well as evaluating the biomarkers' response to the treatment and prognosis.^{1,2} MS is categorized into four clinical forms: relapsing-remitting MS (RRMS), secondary progressive MS (SPMS), primary progressive MS (PPMS), and progressive-relapsing MS (PRMS).^{2,3}

Approximately 87% of patients are presented with RRMS, characterized by acute attacks (relapses) followed by partial or full recovery (remission).³ Studies performed on autoimmune disease biomarkers aim to discover a marker with an abnormal level during the progression and severe stage of the disease and to be normalized during the successful treatment. Such a marker could be used as a tool for detecting and tracking the therapeutic efficacy of drugs. The most useful autoimmune biomarkers are those that could be measured in serum or plasma.⁴ MS is a common progressive neurological disease in young adults that could be accompanied by significant disability which eliminates patients' quality of life. Currently, the prognosis of this disease is more commonly based on clinical information (relapse rate and degree of disability) and diagnostic tests (brain MRI or the presence of oligoclonal bands in cerebrospinal fluid (CSF)). However, determining the accurate prognosis based on this information is limited. A lot of studies have been performed in this field to find the appropriate biomarker for MS.^{5,6} Matrix

metalloproteinases (MMPs) are a family consisting of at least 23 endopeptidases which act in alteration of the extracellular matrix (ECM) under physiological and pathological conditions. MMPs include gelatinases, collagenases, stromelysins, and other MMPs. Their activity is regulated by tissue inhibitor of metalloproteinases (TIMPs), which is consisted of four endogenous antagonists that are bound to active sites of MMPs. There are many pieces of evidence for the key role of MMPs in the pathogenesis of many inflammatory neurological diseases.⁷ Since the MMPs are a group of proteolytic enzymes dissolving the ECM, they help the leukocytes to migrate through the tissues during the inflammation. MMP-9 is one of these enzymes. Studies have shown that in patients with MS, the serum level of MMP-9 is increased during the inflammation and relapse of the disease, and correspondingly, the level of TIMP-1 (which inhibits this enzyme) is reduced.^{8,9} Studies have also shown that the level of MMP-9 is decreased in the serum of interferon beta-treated patients, which decreases the level of disease activity.¹⁰ *In vitro* experiments and the results of MS in animal models showed that MMPs are agents contributing to the destruction of the blood-brain barrier (BBB), the entry of immune cells into the brain parenchyma, the escalation of TNF- α release, and decomposition of myelin proteins.^{7,11} The MMP-9 facilitates the migration of T cells to the CNS; while TIMP-1 inhibits the function of MMP-9.¹² Based on several studies, MMP-9 levels in serum of RRMS patients are high at the acute phase of the disease.¹³ One of the important points that may have been neglected is the importance of studies focusing on the assessment of the validity of these biomarkers for their use in clinics. On the other hand, finding a reliable prognostic could be effective in the starting point for appropriate treatment and changing the course of disease.¹⁴ Certainly, the use of biomarkers that show the patient's level of activity, more specific treatments could be applied. In this study, the serum levels of MMP-9 and its inhibitor, TIMP-1, were investigated in patients with MS who were treated with Saffron pills and placebo.

MATERIALS AND METHODS

Patient Selection and Ethical Consideration

This triple-blinded study was approved in the ethics committee of Tehran University of Medical

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Sciences (ethics number 90/d/130/1887) and registered in the Iranian Registry of Clinical Trials (registration number, IRCT138802091859N1). Forty-three patients were randomly divided into two groups of 22 (group A, placebo) and 21 (group B, Saffron pill) individuals after confirmation of the inclusion criteria and providing consent from all patients. RRMS patients aged between 18-50 years and expanded disability status score (EDSS) between 0-5.5 were included. This study was undertaken in two centres in Iran (Sina Hospital and Brain and Spinal Injury Repair Research Centre in Emam Khomeni Hospital). These individuals were randomly divided into two groups; the intervention group receives Saffron (500 milligrams three times a day, oral) and the control group received a placebo (three times a day, oral). Saffron tablet, was chemically formulated from the stigma of the Saffron plant, (500 milligrams three times a day for 12 months, oral) and made in Drug Unit, School of Pharmacy, Tehran University of Medical Sciences without any generic name. Placebo tablet was filled with the materials used in the preparation of pharmaceutical products (500 milligrams three times a day for 12 months, oral) made in the same centre. To avoid any prejudgment, both patients' groups and physicians were not informed about the type of prescribed medication (Saffron or placebo). Inclusion criteria: 1. Patients with RRMS based on McDonald's Criteria 2. age between 18-50 years old 3. EDSS between 0 and 5.5 4. Undertreatment with Betaferon or Extavia 5. Signing consent form. Exclusion criteria: 1. Being pregnant during the trial or intention to get pregnant at the start of trial 2. Psychiatric disease or major depression 3. Allergy or hypersensitivity reaction to Saffron in the start of trial 4. Treatment with intravenous immunoglobulin (IVIG) within six months before trial 5. Impaired liver function tests (more than three times of normal ranges) 6. Lack of medication for more than four weeks.

Sampling and Biomarkers

Patients in both groups were evaluated and compared in terms of the number of relapses and EDSS at a specific time interval. The blood sample of two groups was collected in Venoject tubes at the MS Research Center of Sina Hospital and venous blood samples were collected at sterile conditions. After centrifuging, the serum was isolated and stored at -70°C. After 12 months of intervention with Saffron pills and placebo, 5 mL of venous blood was sampled again and centrifuged at the same conditions as the

previous stage and the serum was stored at -70°C. For the control group, similar sampling was done. Samples were transferred to the Immunology Laboratory of Tarbiat Modares University in frozen condition. The samples were thawed to measure the biomarker levels. Samples were evaluated based on the instructions of the kits for MMP-9 (Quantikine ELISA Kit, R&D, USA, code number: DMP900) and TIMP-1 (Human TIMP-1 DuoSet ELISA Kit, R&D, USA, code number: DY970-05). Finally, concentration of each biomarker was determined by reading the wavelength of 450 nm using the ELISA reader.

Data Analysis Method

After entering the information in SPSS software, data analysis was done by the epidemiologist and analyzed; using SPSS ver.13. The paired t-test and Likelihood-ratio χ^2 were applied to compare the baseline data to show any significant differences between the two groups. The variables were presented as means \pm standard deviation and the P value less than 0.05 was considered statistically significant. We used Kolomogorov-Smirinov test (K-S) to check the normal distribution of data.

RESULTS

The Results of MMP-9 Assay

Total participants were 43 patients including 22 and 21 patients in groups A and B, respectively. Male to female ratio was 2/20 and 2/19 in groups A and B, respectively. There was no difference based on sex between the two groups ($p=0.192$). (Table 1).

Since the reduction of MMP-9 level could decrease the severity of disease in patients with MS, in this study we measured the serum level of these MMPs in patients with MS before and after treatment with Saffron pills and compared the results with the group of patients who received placebo. The results indicated that the level of MMPs was significantly decreased only in patients taking Saffron pills after 12 months ($p=0.006$). Also, the rate of MMP-9 in patients with MS before and after treatment with placebo pills was insignificant after 12 months (Figure 1).

The Results of TIMP-1 Assay

Since the increase of TIMP-1 level can decrease the severity of disease in patients with MS, in this study we measured the serum level of TIMP-1 in patients with

MS before and after treatment with Saffron pills and compared the results with a group of patients receiving placebo. The results indicated that the level of TIMP-1 was significantly increased only in patients taking

Saffron pills after 12 months ($p=0.0002$). Also, the rate of TIMP-1 in patients with MS before and after treatment with placebo pills was insignificant after 12 months (Figure 2).

Table 1. Baseline characteristics prior to the intervention

Baseline Variables	Total		Trial treatment A		Trial treatment B		p value	Test
	mean \pm Std	[95% CI]	mean \pm Std	[95% CI]	mean \pm Std	[95% CI]		
Age (years)	33.5 \pm 5.84	[31.84-35.16]	32.75 \pm 6.47	[30.02-35.48]	34.19 \pm 5.23	[32.08-36.31]	0.389	t-test
Sex (Female)*	45 (90%)	[77%-95%]	23 (95%)	[73%-99%]	22 (84%)	[64%-94%]	0.172	LR chi ²
Marital status*							0.192	LR chi ²
Single	13 (26%)	[15%-40%]	9 (37.5%)	[20%-58%]	4 (15.38%)	[5.6%-35%]		
Married	35 (70%)	[55%-81%]	14 (58.33%)	[37%-76%]	21 (80.77%)	[60%-92%]		
Divorced	2 (4%)	[0.9%-15%]	1 (4.17%)	[0.5%-26%]	1 (3.85%)	[0.5%-24%]		
BMI (Kg/M ²)	24.74 \pm 4.36	[23.50-25.98]	25.04 \pm 4.38	[23.19-26.89]	24.46 \pm 4.41	[22.68-26.24]	0.644	t-test
MS diagnosis (Months)	69.64 \pm 47.38	[56.18-83.10]	72 \pm 48.20	[51.65-92.35]	67.46 \pm 47.45	[48.30-86.63]	0.739	t-test
MS onset (Months)	95.3 \pm 60.25	[78.18-112.42]	88.17 \pm 49.08	[67.44-108.89]	101.88 \pm 69.32	[73.89-129.89]	0.427	t-test
ARR 1 year prior Trial (Number)	0.76 \pm 0.89	[0.51-1.01]	0.63 \pm 0.88	[0.26-0.99]	0.88 \pm 0.91	[0.52-1.25]	0.310	t-test
ARR two years prior Trial (Number)	0.9 \pm 1.07	[0.60-1.21]	0.79 \pm 1.06	[0.34-1.24]	1 \pm 1.10	[0.56-1.44]	0.499	t-test
Last attack (Number)	23.62 \pm 23.71	[16.88-30.36]	29.17 \pm 24.46	[18.84-39.50]	18.5 \pm 22.24	[9.52-27.48]	0.113	t-test
Last pulse (Number)	31.42 \pm 32.43	[22.00-40.83]	33.45 \pm 28.48	[20.83-46.08]	29.69 \pm 35.91	[15.19-44.20]	0.693	t-test

Std: Standard deviation, CI: Confidence Interval, BMI: Body Mass Index, Kg/M²: Kilograms per Meters², MS: Multiple Sclerosis ARR: Annualized Relapse Rate, LR chi²: likelihood-ratio chi² *n (%). Total participants were 43 patients including 22 and 21 patients in groups A and B; respectively. Male to female ratio was 2/20 and 2/19 in groups A and B; respectively. There was no difference based on sex distribution between two groups ($p=0.192$). Vitamin-D3, Tolterodine, Gabapentin, Citalopram, and Vitamin-B1 were the most common concomitant drugs that were used in group A. Vitamin-D3, Gabapentin, Amantadine, Calcium-D, Vitamin-B1 were the most common concomitant drugs that were used in group B.

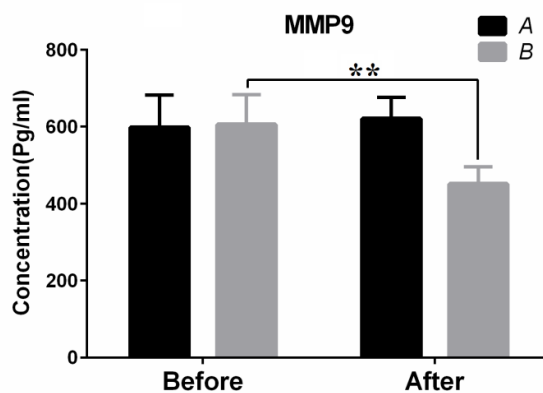


Figure 1. The serum level of cellular MMP-9 matrix metalloproteinases in MS patients was measured; using the ELISA method. Comparison of MMP-9 serum concentration in patients with MS who took Saffron pills (A) and patients who took the placebo (B) before starting treatment (before: Before treatment) with 12 months after treatment (After: after treatment). Paired t-test was used to evaluate the difference in serum level of MMP-9 before and after treatment in each group ($p=0.006$). Data shown as mean \pm SD of A=22(placebo), B=21(Saffron pill) patients; ** $p<0.001$, * $p<0.0001$.**

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MS: Multiple Sclerosis Disease, ELISA: ELISA Test, MMP-9: Matrix metalloproteinase 9, SD: Standard deviation, *p*: *p* value, pg/mL: picogram to milligram

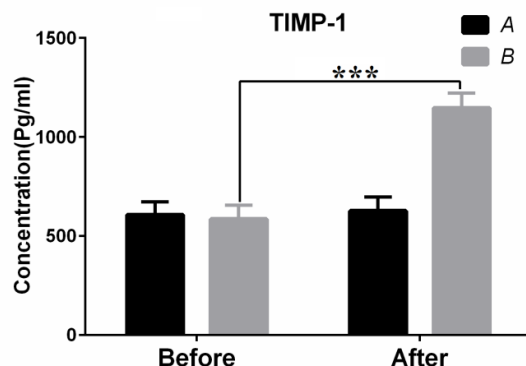


Figure 2. TIMP-1 serum level was measured in MS patients using the ELISA method. Comparison of TIMP-1 serum concentration in MS patients taking the Saffron pills (A) and patients taking placebo (B), before starting treatment (Before: before treatment) or 12 months after treatment (After: after treatment). A paired t-test was used to evaluate the difference in serum TIMP-1 level before and after treatment in each group ($p=0.0002$). Data shown as mean \pm SD of A=22(placebo), B=21 (Saffron pill) patients; ** $p<0.001$, *** $p<0.0001$.

MS: Multiple Sclerosis Disease, ELISA: ELISA Test, TIMP-1: metalloproteinase inhibitor 1, SD: Standard deviation, *p*: *p* value, Pg/ml: picogram to milligram

DISCUSSION

MS is an autoimmune disease that affects the brain and spinal cord characterized by the presence of inflammatory lesions and demyelination. In MS, T-cells originating in the peripheral immune response and eventually infiltrate the CNS; producing demyelination and axon degeneration.¹⁵ The source of autoimmunity in MS remains to be identified. However, anti-inflammatory therapies are efficient in reducing relapse and progression.¹ It is possible that in addition to infiltrating T-cells that attack the myelin, infections that target the brain may activate an inflammatory response that then goes on to attack oligodendrocytes.^{1,2,15} Globally, the median estimated prevalence of MS is 112.0 per 100000 and the median estimated incidence of MS is 5.2 per 100000.¹⁶ Iran is considered as a country with a high MS prevalence (51.52 per 100000) in the Middle East.¹⁷ although most of the activity factors and the cause of the disease are still precisely unclear, some studies have shown that the course of its pathological process includes two aspects: the removal of myelin and the destruction of nerves in the CNS. The distinguished incidence of MS is the formation of plaques that have lost myelin and cause the impairment in the BBB, especially at the

early stages of sedimentation. MMPs have been found to cause tissue damage through two mechanisms: the first mechanism is that they are secreted by active inflammatory cells to break down the endothelial veins so that the inflammatory cells can escape from the blood vessels to the main tissue. In animal studies, it has been proven that preventing the proliferation or activity of T cells and macrophages leads to improve the disease process. The second mechanism is that where the expression of MMPs increases, it can exacerbate the pathological pathway through activation of certain inactive forms of inflammatory mediators, and MMPs break the sheath of myelin in CNS parenchyma. Breaking the products of this proteolytic activity results in more tissue damage by starting a cascade of events that finally causes demyelination and inflammation in the CNS.¹⁸ The integrity of the ECM is preserved by the dynamic equilibrium between the synthesis and proteolysis of their components which is mainly done by MMPs and their inhibitors.¹² TIMP-1 is an MMPs inhibitor induced by cytokines and hormones. Different observations indicate that increasing the level of MMPs is associated with the decrease in TIMP-1 levels which plays a role in the loss of integrity of the BBB through the extracellular brain endothelial proteolysis in MS patients.¹⁹

According to the aforementioned comments and with the development of therapeutic options for MS, there is a growing need for the biomarkers which are more sensitive and can measure the activity and course of the disease. Today, various biomarkers are used with high selectivity and accuracy to help the initial treatment, assess the response to drug treatments, early diagnosis, and the identification of different stages of this disease. Therefore, in this study, the serum level of MMP-9 and its specific inhibitor, the TIMP-1, were measured in patients with MS treated with Saffron tablets and the control group (placebo). In the present study, we found that the levels of MMP-9 and TIMP-1 in the serum of these individuals can be used as a valuable indicator for following the improvement of patients treated with Saffron pills. According to the findings of the current study, the rate of MMP-9 was significantly reduced in patients with MS before and after 12 months of treatment with Saffron pills. Also, the rate of MMP-9 in patients with MS was insignificant before and after 12 months of treatment with placebo pills. Since TIMP-1 is an inhibitor for MMP-9, it has shown a statistically significant increase in the group treated with Saffron pills. In addition, the rate of TIMP-1 in patients with MS was insignificant before and after 12 months of treatment with placebo pills. Previous studies have shown that MMP-9 is not present in CSF of healthy adults and it is increased in inflammatory diseases such as MS. In this regard, serum analysis showed that MMP-9 level in patients with MS was significantly higher than the control group. The results of this study were consistent with the results of Carabodak et al; examining the effect of IFN- β 1a on MMP-9 and TIMP-1 in RRMS patients during one year. They reported that MMP-9 level did not show a significant change; while the TIMP-1 level was gradually increased during treatment which was significantly higher than the pre-treatment levels.¹² Garcia Montujo et al reported a double-fold in MMP9/TIMP1 ratios on 50 MS patients treated with INF-beta for two years. It seems that the MMP9 / TIMP1 ratio may be applied as an indicator of the high vitality of INF-beta.²⁰ In 2002, Luzi et al observed a significant reverse association between MMP-9 and its endogenous TIMP-1 inhibitor in RRMS. This finding suggests that in patients with MS, both increase in MMP-9 and reduction of TIMP-1 play a role in degradation of BBB and the entry of T lymphocytes into the CNS.²¹ The results of the present study were consistent with the results of Correale et al.

and Sastre-Garriga et al reporting that CSF and/or serum MMP-9 amounts are higher in RRMS rather than the progressive forms²². Among patients with active MS, MRI findings showed enhancing lesions compared to those without Gadolinium (Gd), and within patients who had clinically isolated syndromes, definite MS was observed compared to those without disease evolution.²³ Fainardi et al showed that there is a significant reverse relationship between MMP9/TIMP1 and MS activity.²⁴ In 1998, Leppert et al reported that MMP9 expression was significantly increased in the CSF of patients with MS who had experiences recurrence compared to controls.²⁵ In a study by Gray et al, it was shown that active MMP-9 levels were higher in demyelinated than in non-demyelinated or control cortex and the elevated MMP-9 in cortical plaques was associated with loss of perineuronal nets. It was concluded that this elevation of MMP-9 in cortical plaque may lead to neuronal dysfunction and degeneration observed in MS patients. Therapeutic strategies aiming to block the MMPs at both systemic and central levels help to suppress the development process and degenerative events in MS.

Thus, it is unlikely that current treatments prevent inflammatory cells at a systemic level or could prevent the harmful actions of MMP inside the CNS. To advance this gap, future researches should focus on the development of drugs that are readily able to cross the BBB and apply their actions to MMP produced from CNS static cells. The first method in this regard must be the use of combination therapy. By combining the drugs and compounds with different mechanisms of action, they may use their interaction on different inflammatory pathways that are responsible for the disease progression in MMPs activation. Experimental studies have concluded that overproduction of MMPs, lack of activity control and expression are involved in various levels of MS pathogenesis. Therefore, blocking the MMP at the peripheral level may be a valuable therapeutic strategy. The most important limitation of this study was the small sample size due to the lack of cost. Limited duration of the follow-up may influence the result of this trial. Using crocin as the main component of Saffron may have an important impact on serum levels of TIMP-1 and MMP-9. The constraints of the present study could be noted as a lack of cost which resulted in a limited sample size as well as the eliminated number of healthy controls and a short period of follow-up.

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In conclusion, the study finding revealed that Saffron could be prescribed to reduce the serum level of MMP-9 and increase the serum level of TIMP-1 in RRMS. Over production of MMPs and their uncontrolled expression are involved in various levels of MS pathogenesis. Therefore, blocking the MMPs at peripheral levels might be a valuable therapeutic strategy in the management of MS.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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