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The Effects of Mesenchymal Stem Cells on the Gene Expression of TGF-beta and IFN-gamma in Patients with Rheumatoid Arthritis

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ABSTRACT

The therapeutic and immunomodulatory potential of mesenchymal stem cells (MSCs) in rheumatoid arthritis (RA) has attracted considerable scientific attention in recent decades. This study aimed to evaluate the expression of genes encoding interleukin *(IL)4* and *IL10*, as well as interferon-gamma (*IFNG*) and transforming grow*th* factor beta (*TGFB1*) in refractory RA patients following intravenous injection of autologous bone marrow-derived MSCs (BM-MSCs).

This study was registered in Iranian Registry of Clinical Trials (IRCT) (2015102824760N1) and ClinicalTrials.gov (identifier: NCT03333681). Blood samples were taken from 13 patients before and 1 and 6 months after the MSC injection to evaluate the clinical manifestations, paraclinical factors, and expression of *IL4*, *IL10*, *IFNG*, and *TGFB1* genes employing the SYBR Green real-time reverse-transcriptase polymerase chain reaction (RT-PCR) technique.

There was a significant increase in the expression of *TGFB1* at 1 and 6 months after the MSC injection compared to that in the baseline, while the expression of *IL4* and *IL10* did not change significantly. On the other hand, the expression of *IFNG* increased significantly after 1 month but decreased significantly at 6 months compared to 1 month after the intervention. Nevertheless, it showed no significant decrease compared to the baseline.

A significant decrease was observed for the expression of *IFNG* 6 months after the injection compared to that after 1 month, which was in concordance with the rise in the expression of the *TGFB1* gene. A significant change in the gene expression of *TGFB1* and *IFNG* in our study was

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consistent with the amelioration of clinical manifestations, suggesting a mechanism of action for MSCs in the treatment of RA.

Keywords: Cytokines; Immunomodulation; Inflammation; Mesenchymal stem cells; Rheumatoid arthritis

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic, chronic, autoimmune inflammatory disease that causes irreversible and progressive physical dysfunction due to joint destruction. The disease is more prevalent among women, often occurring in the age range of 30 to 50 years. Improper immune system activation leads to loss of immune tolerance, presentation of autoantigens, and activation of self-reactive T and B cells, thus, overproduction of inflammatory cytokines.[1,2]

Paracrine and autocrine functions of cytokines produced by synovial cells can perpetuate inflammation in RA and ultimately lead to cartilage and bone destruction.[3] Helper T (Th) cells differentiate into Th1, Th2, Th17, and regulatory T cells (Tregs) according to the cytokine microenvironment. The active form of RA may be the result of a shift toward a subset of proinflammatory T lymphocytes, predominantly Th17, versus anti-inflammatory Treg. In RA, $CD4^+$ T cells stimulate macrophages, synovial fibroblasts, and chondrocytes to yield proinflammatory cytokines (mainly interleukin *(IL)1β*, *IL-6*, and tumor necrosis factor alpha (*TNFA*)) and activate B lymphocytes. They also stimulate osteoclastogenesis in the bone. Ultimately, these cells lead to joint destruction and progression in RA.[4]

In RA, Th1 cells and their cytokines, especially interferon-gamma (*IFNG*), effectively induce and perpetuate chronic inflammation and tissue destruction. However, evidence has shown that *IFNG*, in addition to its proinflammatory effect, may act as a significant regulator in inflammation and immune responses.[5] Studies have indicated *IL4* as an anti-inflammatory agent for joint injury. Furthermore, it has been reported to prevent bone erosion, improve osteoarthritis, and repair tissue in collagen-induced arthritis (CIA) mouse models.[6] Like IL4, IL10 is a potent cytokine that has anti-inflammatory and immunomodulatory effects. It inhibits the production of proinflammatory cytokines, especially IL1, IL6, and TNFA, which are secreted by activated macrophages and monocytes. Moreover, *IL10* increases mast cell production and prevents the *IFNG* creation with natural killer cells.[7] The role of transforming growth factor beta (*TGFB*) in RA still remains unclear; nonetheless, it appears that this cytokine has immunomodulatory effects.[8]

Over the years, early intervention through disease-modifying antirheumatic drugs and the use of new drugs has resulted in improvements in the symptoms of a wide range of RA patients. Long-term administration of such medications may, however, lead to side effects for a substantial number of patients with RA. Furthermore, numerous RA patients are resistant to such treatments.[9] Therefore, further research on alternative therapies, such as injection of mesenchymal stem cells (MSCs), could be conducive to the management of RA symptoms.

Today, MSC therapy has shown good therapeutic potentials owing to its self-regenerating power and immune-modulating properties. Clinical studies have also revealed satisfactory statistics in terms of MSC treatment in RA patients.[9, 10] MSCs have been shown to inhibit the secretion of *TNFA* with dendritic cells (DCs), and *IFNG* through Th1 and natural killer cells. Moreover, MSCs increase the expression of suppressive cytokines, including the secretion of *IL10* and *IL4* from Th2 cells; hence, MSCs change the path of Th1 to Th2. In other words, they direct the immune response to an anti-inflammatory/immunological tolerance phenotype.[11] In the present research, we aimed to assess the impact of intravenous injection of bone marrow-derived mesenchymal stem cells on the expression of genes encoding *IL4*, *IL10*, *IFNG*, and *TGFB1* in refractory RA patients.

MATERIALS AND METHODS

According to the classification criteria for RA (ACR /EULAR 2010),[12] thirteen patients with RA, who did not react to conventional therapies, were recruited in this clinical trial. During the treatment, they received prednisolone (less than 10–15 mg per day), methotrexate (7.5–25 mg per week), sulfasalazine (less

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than 1–2 g per day), and hydroxychloroquine (less than 400 mg per day).

We employed the RNA samples from the bank archive according to the approvals of ethics committee of Mashhad University of Medical Sciences, Iranian Registry of Clinical Trials (IRCT code: IRCT2015102824760N1), and Clinicaltrials.gov (NCT03333681 identifier).[10,13]

Evaluation of Gene Expression via SYBR Green Real-time PCR

After collecting the whole blood samples before and 1 and 6 months after the injection of bone marrow-derived MSCs (BM-MSCs), peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll and centrifugation. RNA was obtained from PBMCs, and cDNA was synthesized in accordance with the manufacturer's instructions (Yekta Tajhiz Azma kit, Iran, www.yaktatajhiz.com). SYBR Green real-time PCR was performed on 4 µL of the cDNA sample using 5µL of SYBR Green quantitative PCR master mix, 0.4 µL of each primer pair, and 0.2 µl of molecular water (in the total volume of 10 µL). SYBR Green real-time PCR was utilized for evaluating the changes in the expression of genes encoding *IL4*, *IL10*, *TGFB1*, and *IFNG*. The real-time PCR program was as follows: primary denaturation was done for 10 minutes at 95°C. Subsequently, the following steps were repeated for 40 cycles: denaturation for 10 seconds at 95°C, annealing for 30 seconds at 60°C, and elongation of the target sequence at 72°C for 20 seconds. Supplementary Table 1 represents the primer sequence of the glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) and target genes as housekeeping genes.

Statistical Analysis

To compare the data at the 3 time points, including zero, 1, and 6 months after the MSC injection, a generalized estimating equation test was performed using IBM SPSS Statistics 22 software. In all the calculations, $p<0.05$ was regarded as the level of significance.

RESULTS

Demographic, Clinical, and Paraclinical Information of the Patients During the Treatment

The participants were all female, aged 33-58, with a mean age of 44 years. Visual analog scale (VAS) and disease activity score 28-erythrocyte sedimentation rate (DAS28-ESR), as well as anti-cyclic citrullinated peptide (anti-CCP), ESR, rheumatoid factor (RF), and C-reactive protein (CRP), were measured, analyzed, and reported previously by Ghoryani et al.[10,13]

Gene Expression of *IL4, IL10, IFNG,* and *TGFB1*

Herein, the expression of *TGFB1* significantly increased at months 1 (p *value*=0.049) and 6 (p *value*=0.0002) after the MSC injection compared to that in the baseline. Meanwhile, its expression rose, although not significantly after 6 months compared to 1 month after the treatment (p value=0.11) (Table 1 and Figure 1a). Additionally, the *IL10* gene expression increased insignificantly at months 1 and 6 as compared with the baseline (p=0.85, 0.16, respectively) (Table 1 and Figure 1b). *IL4* gene expression also increased at months 1 (p=0.07) and 6 (p=0.56) following the treatment in comparison with the baseline; however, this increase was not statistically significant (Table 1 and Figure 1c). Furthermore, there was a significant rise in the expression of *IFNG* at month 1 compared to that in the baseline (p=0.02). However its expression was significantly reduced at month 6 compared to month 1 (p=0.03). In comparison with the baseline, its expression showed an insignificant decrease after 6 months (p=0.51) (Table 1 and Figure 1d).

Table 1. Relative gene expression (mean±SEM) of interleukin (*IL)4*, *IL10*, interferon-gamma (*IFNG)*, and transforming growth factor beta (*TGFB1)* in patients with refractory rheumatoid arthritis before the mesenchymal stem cell transplantation (MSCT) and 1 and 6 months after the intervention

Gene	ΔCt Before MSCT	ΔCt 1 month After intervention	ΔCt 6 months After intervention	*p* (between time intervals) 0-1	0-6	1-6
TGFβ1	−11.75±0.63	−10.01±0.80	−8.38±0.66	(*p*=0.049)	(*p*=0.0002)	*p*=0.11)
IL10	−11.17 ±0.69	−10.95±1.00	−10.12±0.29	(*p*=0.85)	(*p*=0.16)	-
IL4	−11.42±0.34	−10.50±0.23	−10.92±0.77	(*p*=0.07)	(*p*=0.56)	-
IFNG	−11.84±0.32	−10.55±0.49	−12.27±0.57	(*p*=0.02)	(*p*=0.51)	(*p*=0.03)

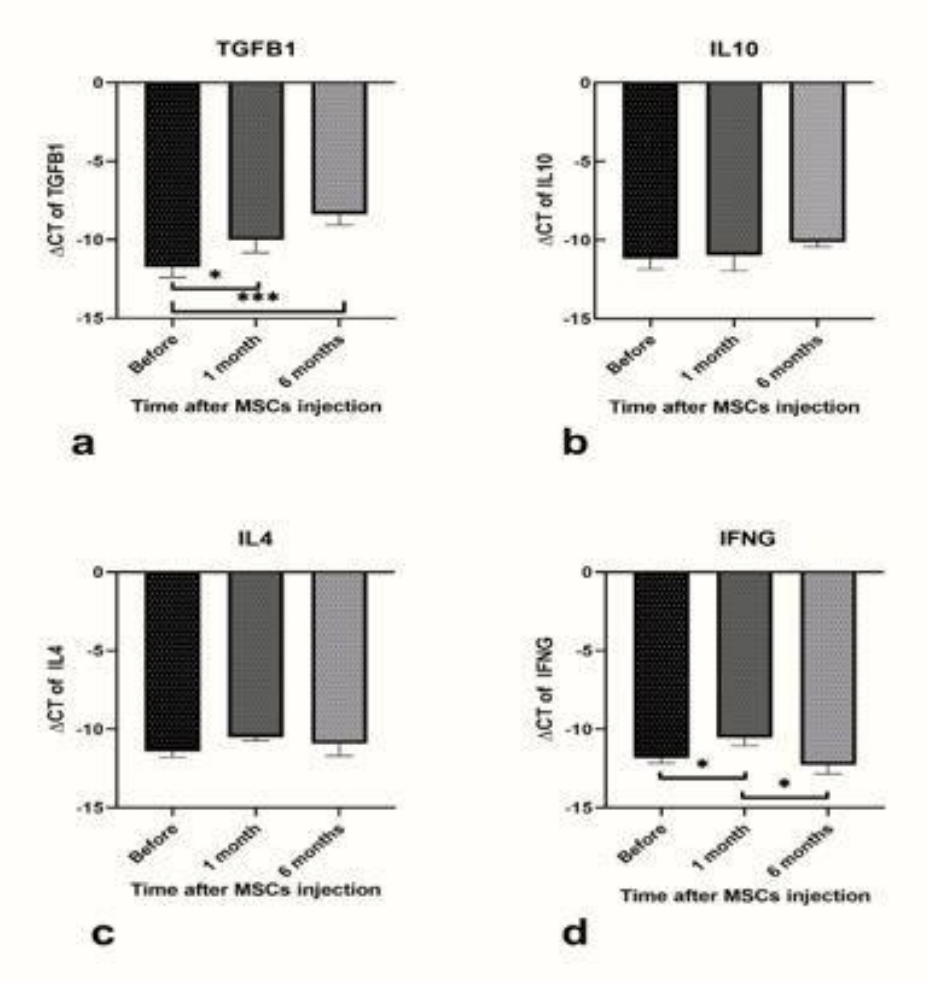

Figure 1. Relative gene expression of transforming growth factor beta (*TGFB1)* (a), interleukin (*IL)10* (b), *IL4* (c), and interferon-gamma (*IFNG)* (d) in patients with refractory rheumatoid arthritis, before the injection of bone marrow-derived mesenchymal stem cells, 1 and 6 months after. (p*<0.05, ****p*<0.001)**

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DISCUSSION

RA is a chronic, systemic, autoimmune inflammatory condition, which, in addition to clinical symptoms, causes pain, swelling, joint stiffness, and fever, along with multiple-organ disorders.[1, 2] The imbalance between Tregs and Th17 cells is one of the effective mechanisms causing RA.[4] Conventional treatments of RA could lead to several adverse effects, and some patients have shown resistance against these treatments.[9] Thus, further research on alternative therapies, such as MSCs, could contribute to appropriately taking the disease under control and treating it. The current work examined the impact of intravenous injection of BM-MSCs on the expression of *IL4*, *IL10*, *IFNG*, and *TGFB1* in patients with RA.

Previous studies have reported no side effects following MSC therapy in patients with RA.[10,14-16] The results obtained herein revealed an improvement in the severity of inflammation and disease symptoms after intravenous injection of MSC. We also demonstrated that clinical parameters, including VAS, DAS28, and serum levels of RF, significantly decreased, which is in accordance with the results of previous human studies and animal model investigations.[10,13,14,17]

Our findings indicated that in patients with RA, the *TGFB1* gene expression significantly increased by the end of 6 months after the intervention with injection of BM-MSCs as compared with the baseline. There are controversial findings regarding the role of *TGFB1* in various studies of experimental arthritis. In one paper, RA was induced through intraperitoneal injection of group A streptococcal cell wall fragments in an animal model. Following the injection of anti-*TGFB* antibodies, acute and chronic arthritis improved significantly.[18] Moreover, previous studies have shown the pathogenic effects of *TGFB* on synovial tissue, leading to arthritis and synovial hyperplasia after the injection of *TGFB1* in healthy mice.[19,20] On the other hand, several papers report that intraperitoneal and topical administration of anti-*TGFB* antibodies increased the severity of arthritis and the levels of proinflammatory cytokines in animals with type II CIA.[21,22] In addition, the regulatory role of *TGFB* in disease activity and the improvement of the inflammatory process in the CIA have been demonstrated.[23] Another study by Park et al. on CIA mice showed that the injection of *TGFB*–transduced MSCs prevented joint inflammation and the development of arthritis.[24]

The decreased levels of *IL6*, *IL1B*, and *TNFA* and the increased levels of *TGFB* and *IL10* have been observed after the injection of MSC to the CIA mouse models.[17] Similarly, some other papers have shown that combination therapy with MSC and *IL4* reduced joint inflammation, the number of synovial cells, proinflammatory cytokine levels, angiogenesis, and bone destruction in CIA animal models. Moreover, there was a significant decrease in the levels of *TNFA* and monocyte chemoattractant protein-1 (MCP-1), whereas a significant increase was found in *IL10*.[6] Our results implied that the injection of MSCs increased the *IL10* gene expression, although it was not significant.

According to previous research, the increase in the levels of *IL4* and *IL10* prevents inflammation and joint destruction in RA.[25] Zheng et al. stated that MSCs inhibited the response of CII-stimulated T cells in RA patients even after differentiation. They also reported that MSCs could inhibit the production of *IFNG* and *TNFA* by suppressing the effects on T-cell proliferation, thereby ameliorating arthritis inflammatory symptoms. Furthermore, this research indicated an improvement in the efficacy of MSCs with the increase in the level of *IL10* and regulation of *IL4* and *TGFB* secretion.[26] In our study, *IL4* gene expression increased during MSC therapy compared with the baseline, although not significantly. *IL10* and *IL4* gene expression may reach a significant level with the increase in the follow-up period or replicating the injection of MSCs.

The results of another study showed a significant but transient rise in the level of serum *IFNG* in RA patients who responded well or moderately to MSC transplantation. Additionally, an increase in the level of *IL10* and the ratio of Treg / Th17 was observed 2-3 weeks following a transient rise in *IFNG*, whereas *IL6* levels decreased.[16] Moreover, it was shown that the increased *IFNG* serum *IFNG* before and after MSCT was directly associated with decreased DAS28 levels in RA patients.[16] On the contrary, in another paper, *IFNG* inhibited the inflammatory response by suppressing Th17 cell differentiation and *IL17* production.[27] Herein, *IFNG* gene expression increased significantly after 1 month of intervention with MSC injection. Previous studies have shown that *IFNG* is also produced from other sources, such as macrophages and dendritic cells.[28] In our study, the expression of the *IFNG* gene revealed a significant decrease 6 months after the MSC injection compared to that 1 month after. A nonsignificant decrease in *IFNG* gene expression was also detected at

month 6 compared with the baseline. The *IFNG* gene expression significantly decreased 6 months after the injection compared to after 1 month, which was in line with the increased expression of the *TGFB1*. As *TGFB* is one of the important secreted cytokines from Tregs, this reduction in *IFNG* gene expression might be due to the immunomodulatory effects of Tregs on the immune responses boosted via immunomodulatory properties of MSCs.

The effects of MSCs, based on previous clinical studies, were not reported to be permanent, and the swelling and joint pain reoccurred in patients 24 weeks after MSC transplantation. Therefore, a second MSC injection could be suggested after 6 months in order to enhance the effects of MSCs.[16]

According to the results of the present study, success in the treatment of patients with RA with BM-MSCs might be owing to the effect of MSCs on Tregs and the increase in *TGFB1* expression from one side along with the decrease in *IFNG* expression from the other side over 6 months of follow-up of the patients.

Ghoryani et al. indicated the safety and efficacy of autologous MSC transplantation in patients with RA.[13, 10] As our data were based on the RNA sample bank made by these groups, we conclude that MSC therapy may help improve the regulatory effect of Tregs and inhibit the inflammation caused by Th1 cells, which is in concordance with amelioration of the clinical symptoms. Further studies are necessary to find more details of this proposed mechanism, thereby reaching a better conclusion.

STATEMENT OF ETHICS

This trial was accepted by ethics committee of Mashhad University of Medical Sciences (IR.MUMS.MEDICAL.REC.1398.005).

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CONFLICT OF INTEREST

The authors confirm that there are no conflicts of interest to declare.

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