ORIGINAL ARTICLE

Intravenous injection of autologous bone marrow-derived mesenchymal stem cells on the gene expression and plasma level of CCL5 in refractory rheumatoid arthritis

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Background: Rheumatoid arthritis (RA) is the most prevalent autoimmune disease, in which CCL2 and CCL5 are critically involved. The objective was to evaluate the therapeutic effects of bone marrow-derived mesenchymal stem cells (MSCs) on the foregoing chemokines in RA patients. **Materials and Methods:** Thirteen RA patients were evaluated in terms of clinical manifestations, paraclinical factors, gene expression, and plasma levels of CCL2 and CCL5 prior to treatment and 1 and 6 months after intervention. Real-time-polymerase chain reaction and enzyme-linked immunosorbent assay were employed to assess the gene expression and plasma levels of CCL2 and CCL5 at different time points after MSC therapy. Statistical analysis was performed by SPSS 16 and Prism 7. **Results:** The CCL2 gene expression had statistically significantly increased ($P = 0.034$), and its plasma level had insignificantly reduced after 1 month. Furthermore, the gene expression and plasma level of CCL5 had statistically significantly decreased ($P = 0.032$, $P < 0.001$). The CCL5 gene expression had statistically significantly increased after 6 months ($P = 0.001$) and its plasma level had insignificantly reduced. **Conclusion:** The most significant inhibitory effects of MSC therapy on the gene expression and plasma level of CCL5 were observed at the end of 1 month. The differences between the gene expression and protein levels during the treatment might be related to microRNA effects or the insufficient number of MSC injection.

Key words: Chemokine CCL2, chemokine CCL5, mesenchymal stem cells, rheumatoid arthritis

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INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune inflammatory disease accounting for 0.5%–1% of the adult population around the world. The onset of the disease typically occurs at the age of 22–55 years accompanied by synovial inflammation and progressive joint, cartilage, and bone destruction. The main cause of the disease has not been determined yet. However, immunity, genetics, environmental factors, and infections might be involved in the occurrence of the disease.[1]

RA is an inflammatory autoimmune disease in which immune cells and inflammatory mediators play a

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pivotal role. B-cells or T-cells, monocytes, and neutrophils infiltrate the inflammation sites along with inflammatory mediators, such as cytokines and chemokines, having a key role in the occurrence of arthritis. CCL2 and CCL5 generated by fibroblasts, endothelial cells, smooth vascular cells, monocytes, T-cells, and other cell types are among the chemokines involved in the chemotaxis of immune cells to the sites of inflammation, angiogenesis, and osteoclastogenesis.[2-5] Previous studies showed an increase in the gene expression and protein level of two chemokines, namely CCL2 and CCL5, in the serum, joint fluid, and cartilage of RA patients.[6-8]

Chemokines, such as CCL2 and CCL5, produced by M1 macrophage in the synoviocytes, are among pre-angiogenic factors. They stimulate the production of matrix metalloproteinase 3 by chondrocytes and lead to the destruction of cartilage and angiogenesis. Moreover, CCL2 and CCL5 are involved in the activation of osteoclastogenesis and the destruction of cartilage and joints by the migration of osteoclasts to inflamed sites.[4,5]

The primary goals of RA treatment are to prevent or control progressive destruction and eliminate the pain. According to relevant literature, disease recovery is determined by Disease Activity Score 28 (DAS-28). DAS28-erythrocyte sedimentation rate (ESR) or DAS28-C-reactive protein (CRP) was employed as a primary evaluation tool for RA diagnosis. Today, medical treatments for such patients mainly include nonsteroidal anti-inflammatory drugs, intra-articular glucocorticoids, and disease-modifying anti-rheumatic drugs (DMARDs).[1,9] Because joint injuries do not usually heal in some patients with these common drugs, it is necessary to try new therapies such as stem cell therapy to prevent the disease progress.

Stem cells are nondifferentiated cells capable of proliferation in the culture environment and differentiation into certain cells. In addition, these cells are vital for growth, development, survival, and the repair and construction of bones, muscles, nerves, and other body limbs.[10] Mesenchymal stem cells (MSCs) can inhibit and regulate the immune system in different ways: MSCs inhibit TCD4+ through generating prostaglandin E2 (PGE2), transforming growth factor-β (TGFβ), IDO, and inducible nitric oxide synthase, consequently reducing the proliferation and antibody production of B-cells. MSCs further decrease the production of IFN-γ and increase interleukin-4, thereby causing a shift from TH1 (pre-inflammatory) to TH2 (anti-inflammatory).[11,12]

SUBJECTS AND METHODS

Selection of patients

According to the revised American College of Rheumatology criteria, we enrolled 13 refractory RA patients who did not respond to common medicines. The disease activity was assessed by the DAS-28-ESR. All the 13 RA patients were evaluated with regard to clinical manifestations, paraclinical factors, gene expression, and plasma levels of CCL2 and CCL5 before treatment and 1 and 6 months after intervention. All patients continued to receive DMARDs such as methotrexate, hydroxychloroquine, and sulfasalazine and corticosteroid drugs such as prednisolone during the intervention.

Sample preparation

In this single-arm, clinical trial study, our team performed the intravenous injection of bone marrow (BM)-derived MSCs at an immunology research center in Mashhad University of Medical Sciences to treat patients with refractory RA.[13] The plasma and RNA samples of all patients were recruited from our biobank, which was previously created upon the approved clinical trial registered in the Iranian Registry of Clinical Trial (IRCT) and clinical trial.gov with codes IRCT2015102824760N1 and identifier: NCT03333681, respectively.

SYBR green real-time polymerase chain reaction for gene expression assay

Peripheral blood samples were collected before and 1 and 6 months following the intervention. Total RNA extraction and cDNA preparation were performed according to the manufacturer's instructions (Yekta Tajhiz Azma kit, www.yaktatajhiz.com). Afterward, cDNA was determined by real-time-polymerase chain reaction (PCR) using SYBR Green Master Mix (www.Takarabio.com) with forward and reverse primers (www.afragen.com) for forty cycles. PCR temperature conditions are mentioned in Table 1. A human glyceraldehyde-3-phosphate dehydrogenase GAPDH was used as an endogenous control for sample normalization. Table 2 summarizes the PCR primers.

Enzyme-linked immunosorbent assay

The plasma levels of CCL2 and CCL5 were determined by enzyme-linked immunosorbent assay (ELISA) (www.biolegend.com), according to the manufacturer's instructions. Values were calculated as mean ± standard error of mean (SEM). The limitation of ELISA assay for both chemokines was between 7.5 and 500 pg/ml.

Statistical analysis

Values were calculated as mean ± SEM. Generalized estimating equation was employed to analyze the differences

Table 1: Polymerase chain reaction temperature conditions

Forty cycles for PCR	Temperature and Time
DNA denaturation	95° for 10 s
DNA annealing	60° for 30 s
Extension	72° for 20 s

PCR=Polymerase chain reaction

Table 2: Primer sequence of GAPDH, CCL2, and CCL5 genes

	GAPDH	CCL2	CCL5
Forward primer	5'CACTAGGCGCTCACTGTTCTC-3'	5'AAACTGAAGCTCGCACTCTCG-3'	5'CCTGCTGCTTTGCCTACATTGC3'
Reverse primer	5'CCAATACGACCAAATCCGTTGAC-3'	5'TTGATTGCATCTGGCTGAGCG-3'	5'ACACACTTGGCGGTTCTTTCGG3'

in gene expressions and plasma levels of chemokines in refractory RA patients before and after the intervention. Statistical analysis was conducted by SPSS 16 (SPSS Inc. Chicago, Illinois, U.S.A) and Prism7 (Prism Systems, Inc. U.S.A). All $P < 0.05$ were considered statistically significant.

RESULTS

Clinical scores and paraclinical laboratory tests

All the 13 patients were women aged 44 ± 2.08 years. Based on the clinical scores, including visual analog scale (VAS) and DAS-28-ESR, our data showed that the disease was improved at the end of the intervention. In addition, rheumatoid factor (RF), CRP, ESR, and anti-cyclic citrullinated peptide (anti-CCP), as paraclinical factors, were significantly reduced at the end of MSC therapy. Please see the details in our recent publication.[13]

Evaluation of CCL2 and CCL5 gene expressions in peripheral blood mononuclear cells of rheumatoid arthritis patients using SYBR green real-time-polymerase chain reaction technique before and after intervention with autologous mesenchymal stem cells

The gene expression of CCL2 and CCL5 and the fold changes were analyzed according to the double delta Ct method. As observed in Figure 1a, 1 month into the treatment, there was a statistically significant increase in the gene expression of CCL2 compared to the baseline ($P = 0.034$). However, this increase was not statistically significant after 6 months. The gene expression of CCL5 in peripheral blood cells was assessed prior to the treatment and 1 month and 6 months after the treatment. This gene expression statistically significantly decreased after a month ($P = 0.032$). However, the gene expression of CCL5 statistically significantly increased after 6 months compared to the baseline and a month into the treatment ($P = 0.001$ and $P < 0.001$, respectively) [Figure 1b].

To calculate the fold changes, the $2^{-\Delta\Delta CT}$ method was employed, and the cutoff point was considered 1. The fold changes for CCL2 after one 1 and 6 months were 1.6 ± 1 and 1.8 ± 1.6, respectively. According to the 2^-ΔΔCT method, the gene expression of CCL2 increased by 60% after 1 month. An increase of 80% was observed in CCL2 after 6 months [Figure 1c], and the fold change of CCL5 was 0.23 ± 0.8 following a month. The gene expression of CCL5 decreased by 77% after 1 month. However, this change was 5.22 ± 1.3 at the end of 6 months, indicating an increase of more than 5 folds [Figure 1d].

Evaluation of the plasma levels of CCL2 and CCL5 in patients with rheumatoid arthritis before and after intervention with autologous mesenchymal stem cells

According to Figure 1e, no statistically significant reduction was found in the plasma levels of CCL2 after 1 month of treatment as compared to the baseline ($P = 0.316$). However, this level increased (though not significantly) at the end of 6 months ($P = 0.06$). CCL5 plasma levels had statistically significantly reduced at the end of 1 month ($P < 0.001$), with no statistically significant differences between 1 and 6 months ($P = 0.226$) [Figure 1f].

Correlation of CCL2 and CCL5 plasma levels with clinical factors in rheumatoid arthritis patients before and after intervention with autologous mesenchymal stem cells

CCL2 and CCL5 had no correlation with any of the clinical factors except for ACPA, which had a positive correlation 6 months into the intervention ($P = 0.03$). Pearson's and Spearman tests were employed to analyze the correlations.

DISCUSSION

RA is an autoimmune inflammatory disease that progressively destroys cartilage and joints. Factors such as chemokines play a critical role in disease progression.[1] Because certain patients show resistance to conventional drugs, many researchers have recommended taking the advantage of MSCs in treating patients with RA.[14-16] In the present study, we explored the effect of MSCs on the gene expression and plasma levels of CCL2 and CCL5 in patients with refractory RA. It was shown that (1) MSC therapy was able to reduce the severity of RA; (2) despite increased gene expression, the plasma level of CCL2 was reduced (though not significantly) at the end of 1 month; (3) both the gene expression and plasma level of CCL5 significantly decreased at the end of 1 month; and (4) no side effects were observed in the patients after the intervention. Previous studies showed improvement in the clinical presentation of RA disease following MSC therapy, which is in line with our results.[13,17] Of note, the source of MSCs in previous studies was either BM or umbilical cord and adipose.[14-16] Our study revealed that treatment with BM-MSC caused a significant reduction in DAS-28, VAS pain score, and RF factors. The previous studies showed that treatment with MSCs derived from the umbilical cord could significantly reduce disease activity, ESR, CRP, and RF 1 and 3 months following the injection. Several studies have reported that VAS factor has a significant positive correlation with the number of monocytes infiltrating the

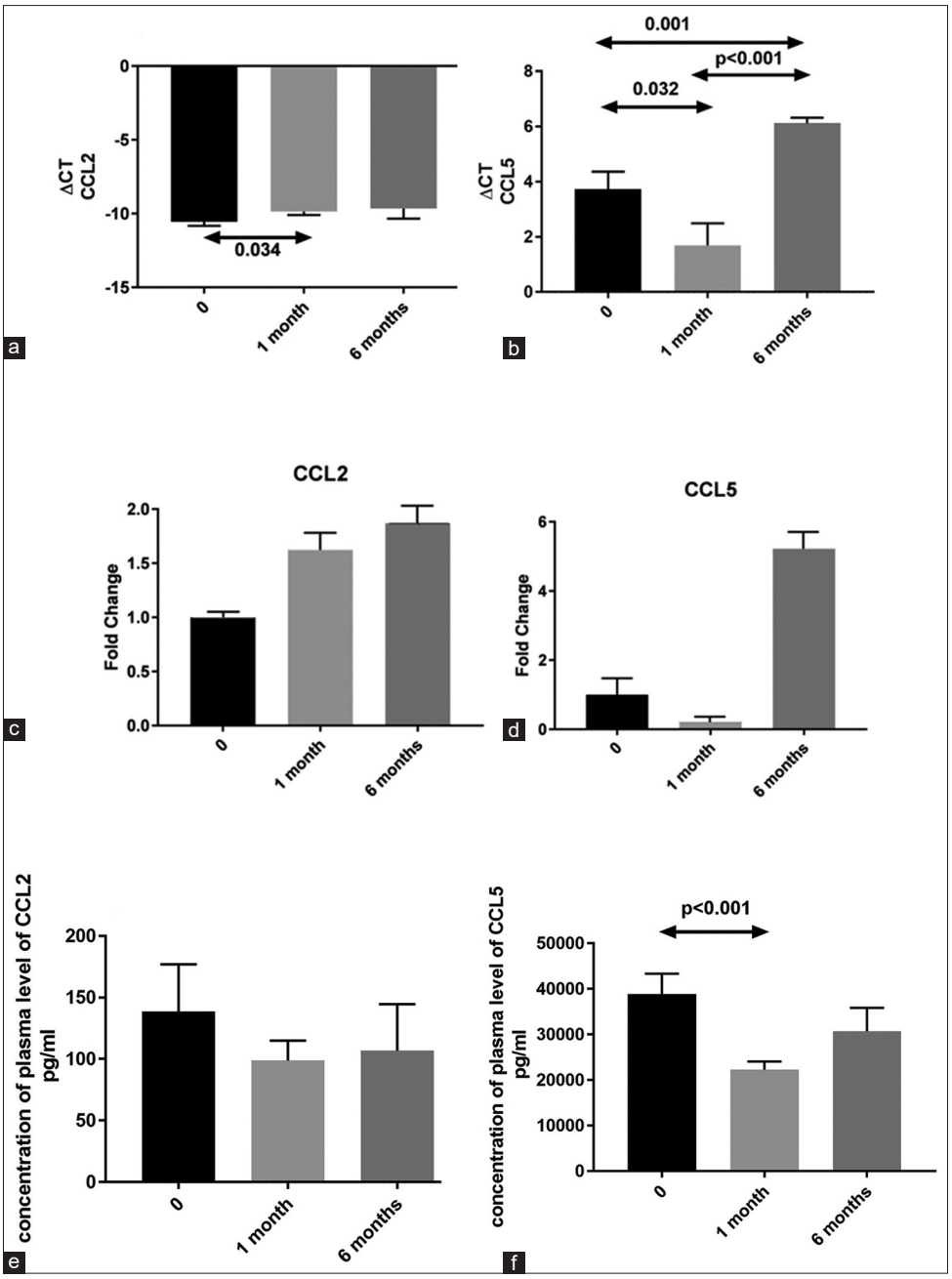

Figure 1: Gene expression (a and b), fold changes (c and d), and plasma levels (e and f) of CCL2 and CCL5 before and 1 month and 6 months after intervention in rheumatoid arthritis patients with autologous mesenchymal stem cell injection

inflammatory site. It is also worth mentioning that MSCs are capable of reducing the number of monocytes. Thus, it was concluded that the reduced disease severity and RF levels in other studies might be ascribed to the suppressive effects of MSCs on monocyte infiltration into the inflamed joints of RA patients and the inhibitory role of these cells concerning B-cell RF production.[16,18] Our study revealed a positive relationship between CCL5 and ACPA 6 months after the intervention. Behrens *et al*. reported that both anti citrullinated protein antibody (ACPA) and CCL5 chemokine were decreased in arthritic DQ8 mice by anti CD-20 treatment.[19]

Myriad studies have reported increased gene expression and plasma levels of CCL2 in RA patients in comparison to healthy individuals.[8,20]

CCL2 and CCL5 are inflammatory chemokines generated from T cells and monocytes.[2] According to the recent publications, MSCs have the ability to inhibit the activity of T cells through either physical interaction or soluble factors.[11] Furthermore, Lee *et al*. demonstrated that treatment by MSCs in MRL/FAS^{Lpr} mice enhanced the clinical symptoms and inhibited T cell function *in vitro* through producing soluble factors, such as No, PGE2,

TGFβ, and IDO.[21] In a similar study, MSCs co-cultured with monocytes were able to inhibit alloreactive T cells.[11] Our study provided evidence that the reduced plasma levels of CCL2 and CCL5 1 month after intervention were presumably caused by the inhibition of T cell production through MSC injection. On the other hand, Shadidi *et al*. proposed that CCL2 and CCL5 play a significant role in the recruitment of T cells and osteoclasts toward inflamed sites.[22] It is known that immunity cells such as T cells and osteoclast, a kind of monocyte/macrophage, are critically involved in RA pathogenesis.[23,24] Our results show that the reduction in the plasma levels of CCL2 and CCL5 1 month after the intervention and the ameliorated severity of RA are probably the result of the decrease in the ratio of T cells and osteoclast migration to inflammation sites.[4] Noteworthy, although CCL2 and CCL5 are known inflammatory factors in the immune system, they are very important agents in recruiting MSC to inflamed sites and causing the interaction between T cells and MSCs to inhibit T cell activation.[25] In addition, the increase in the gene expression and plasma levels of CCL2 and CCL5 at the end of 6 months compared with 1 month might be related to microRNA-155. This regulatory factor is responsible for transcription upregulation and translation of CCL2 and CCL5 chemokines in the CD14 + monocytes of RA patients *in vitro*. Elmesmari *et al*. demonstrated that the increased miR-155 caused by the expression of CCL2 and CCL5 was consistent in peripheral blood mononuclear cells, especially CD14+ monocytes.[26] Another reason for this increase might be the insufficient number of MSC injection in our study. A previous animal model study showed that repeating the MSC injection improved heart infarction.[27] Similarly, in Crohn's disease, during the treatment procedure, umbilical cord-derived MSCs were injected four times per week, as a result of which, the disease was improved within 12 months in these patients.[28] Therefore, repeating the injection might be more effective in controlling inflammatory conditions.

In the present study, a significant reduction was detected in the gene expression and plasma level of CCL5 at the end of 1 month. A significant increase in CCL5 gene expression and an insignificant decrease in its plasma level were further observed at the end of 6 months. This discrepancy might be explained by the regulatory role of microRNAs in gene translation and expression. Similarly, Nakamachi *et al*. and Wei *et al*. reported that mir124a and mir33 regulated CCL2 gene translation in synoviocyte and chondrocyte, respectively.[29,30] In addition, Hong *et al*. showed that mir200-c is one of the posttranscription regulatory factors which directly target the 3UTR of CCL5 and miR-200; this factor might also be potentially considered as effective in preventing inflammation and osteoclastogenesis and enhancing bone regeneration.[31]

CONCLUSIONS

To our knowledge, the present study is the first to attempt at treating refractory RA patients by MSC therapy with the aim of studying the effects of MSCs on gene expression and the protein levels of CCL2 and CCL5. The effect of MSCs CCL5 plasma level reduction was most optimal at the end of 1-month intervention. The differences between the gene expression and protein levels during the treatment might be attributed to microRNA effects or the insufficient number of MSC injection. More than one time injection of MSCs might be a good approach to controlling inflammatory conditions and chemokine production. Our results provide novel and valuable information concerning the immunopathogenesis of RA disease and the influence of MSCs on chemokines.

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Conflicts of interest

There are no conflicts of interest.

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