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RESEARCH ARTICLE

The Situation of Chemokine Ligands and Receptors Gene Expression, Following the Oral Administration of Drug Mannuronic Acid in Rheumatoid Arthritis Patients

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Abstract: ***Background****:* Regarding the leukocytes infiltration into the synovium of Rheumatoid Arthritis (RA) patients is mostly mediated by chemokine ligands and receptors, and following the efficient and motivating results of international Phase III clinical trial of β-D-Mannuronic acid (M2000) patented EP067919 (2017), as a novel anti-inflammatory drug, in patients with RA, the present research was designed.

Objectives*:* This study aimed to assess the oral administration effects of this new drug on gene expression of some chemokine receptors and ligands, including CXCR4, CXCR3, CCR2, CCR5 and CCL2/MCP-1 in PBMCs of patients with active form of RA.

Methods*:* Twelve patients suffering from RA, with inadequate response to conventional drugs were selected (Clinical trial identifier IRCT2017100213739N10) and 1000mg/day of M2000 was orally administrated to them for 12 weeks. The mRNA expression of target molecules was then evaluated in PBMCs of the patients before and after treatment with M2000 using real-time PCR and was compared to healthy controls. Patents related to this study were also reviewed.

Results*:* The results showed that M2000 was able to significantly down-regulate the mRNA expression of CXCR4, CCR2 and CCL2/MCP-1 in the PBMCs of the RA patients. It should be noted that the gene expression situation of the target molecules was in coordinate with the clinical and paraclinical assessments in the patients.

Conclusion*:* Taken together, the results of this investigation revealed the part of molecular and immunological mechanisms of drug Mannuronic acid (M2000) in the treatment of RA, based on chemokine ligands and receptors mediated processes.

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1. INTRODUCTION

Rheumatoid Arthritis (RA) is a chronic autoimmune disorder, leading to joint deformity, accompanied by cartilage and bone destruction. RA is characterized through a high serum level of autoantibodies, joints swelling, morning stiffness, fatigue and pain [1-5]. It is determined that about 1% of the world's population suffers from this disease, and its prevalence in women is two or three times more than in men [4, 6]. Although the leading cause of RA has not been identified yet, however, many studies have shown that different genetic, epigenetic and environmental factors play a role in RA incidence [7-10]. Vast infiltration of immune cells, including T and B lymphocytes, monocytes and Dendritic Cells (DCs) is occurred into the Synovial Tissues (STs) of

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patients with RA, mostly mediated by chemokine ligands and receptors, and ultimately leading to chronic inflammation and disease exacerbation [2-5, 11]. High amounts of chemokines and their receptors are produced in Peripheral Blood (PB) and Synovial Fluid (SF) of RA patients [11-14]. On the other side, following the ligation of CC and CXC chemokine receptors with appropriate chemokine ligands, the synergic effects can occur between chemokines, which lead to severe infiltration and accumulation of inflammatory cells into the synovium, followed by an intense inflammation in STs [15]. The C-X-C motif chemokine receptor type 4 (CXCR4) has a crucial role in the immunopathogenesis of RA. It is expressed by T and B cells, monocytes and all subpopulations of DCs. CXCR4 interaction with C-X-C motif chemokine Ligand 12/ Stromal cell-Derived Factor 1 (CXCL12/SDF-1), as its major ligand can proceed synovial angiogenesis [12, 16-19]. The C-X-C motif chemokine Receptor type 3 (CXCR3) is the other vital chemokine receptor for leukocytes infiltration in RA, which is expressed by different types of PB and SF inflammatory cells such as effector T and B lymphocytes, monocytes/ macrophages, DCs and memory T cells [12, 16, 17, 19-21]. Its main ligands, including C-X-C motif chemokine Ligand 9/ Monokine Induced by Gamma interferon (CXCL9/MIG) and C-X-C motif chemokine Ligand 10/ Interferon-inducible Protein 10 (CXCL10/ IP-10) are abundantly produced by macrophages and fibroblast-like synoviocytes, which in turn provoke the migration of CXCR3 expressing cells [12, 22]. The C-C chemokine Receptor type 2 and 5 (CCR2, 5), which are expressed by a variety of inflammatory cells including monocytes/ macrophages, T and B cells as well as DCs, also play an important role in RA inflammation. Furthermore, CCR5 gene polymorphism can affect RA incidence and disease phenotype [11, 12, 16, 21, 23-25]. The most principal ligand of CCR2 is C-C motif chemokine Ligand 2/ Monocyte Chemoattractant Protein 1 (CCL2/MCP-1), which is produced essentially by monocytes/ macrophages and plays a critical role in synovial angiogenesis and inflammatory cells recruitment [12, 13, 21, 26].

Along with the Disease-Modifying Anti-Rheumatic Drugs (DMARDs) such as Methotrexate (MTX) patented WO2016067024 (2016) [27], Sulfasalazine (SSZ) patented WO2019101903 (2019) [28], Hydroxychloroquine (HCQ) patented WO2019165337 (2019) [29] and Leflunomide patented WO2014096464 (2014) [30], which are the choice medications in RA treatment for controlling the progression and complications of the disease, the Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) such as Diclofenac patented US20170319484 (2017) [31], Piroxicam patented US20140371211 (2014) [32], Celecoxib patented WO2016196085 (2016) [33] and Naproxen patented WO2017062027 (2017) [34], corticosteroids such as Prednisolone (PRD) patented US20130143853 (2013) [35] and Dexamethasone patented US20190183907 (2019) [36] as well as biologic response modifiers are used in this disease. In spite of the anti-inflammatory effects of all these therapies, some patients show an inadequate response to these conventional drugs. On the other side, they have extensive adverse effects, including gastrointestinal and renal disorders, cardiopathy heart failure, damaging blood cells and osteoporosis. Therefore, designing an effective treatment with very low adverse effects is a vital and substantial target in RA therapy. Drug β -D-Mannuronic acid (M2000) patented EP067919 (2017) [37] is one of the newest members of NSAIDs family with a very low molecular weight (194.139 Da), which many studies have confirmed its anti-inflammatory and immunosuppressive properties, during the years 2004 until now [38-41]. In this connection, the results of international multicenter Phase III clinical trial of Mannuronic acid (M2000) in RA patients showed a potent efficacy following the oral administration of this new drug, which had no to low side effects and it was even able to modify the side effects of concomitant conventional drugs in patients [41]. Based on the above mentioned data, in this study, we aimed to evaluate a part of molecular mechanism of this new drug, through assessing the mRNA expression of CXCR4, CXCR3, CCR2, CCR5 and CCL2/MCP-1, following the oral administration of M2000 by patients with active form of RA, who had shown inadequate response to conventional drugs.

2. MATERIALS & METHODS

2.1. Ethics Approval

This investigation was accepted by the Ethics Committee of Mashhad University of Medical Sciences (MUMS) (No.IR.MUMS.fm.REC.1396.309, Mashhad, Iran) and trial registration number IRCT2017100213739N10 was then obtained. The study was conducted based on the American College of Rheumatology (ACR) criteria [42] and Helsinki manifest guidelines. Written informed consent was signed by All the Enrolled Patients and Healthy Controls.

2.2. M2000 Production and Validation

According to the method of Fattahi *et al*. (2015) [43], Mannuronic acid (M2000) with the chemical formula ($C_6H_{10}O_7$) was extracted from sodium alginate) (Sigma-Aldrich, St Louis, MO, USA) in Immunology Department of Tehran University of Medical Sciences. Afterward, the purity and quality of extracted M2000 were confirmed by determining the status of hydrolytic products using Fourier Transform Infrared (FTIR) and Carbon-13 Nuclear Magnetic Resonance (^{13}C-NMR) spectroscopy methods.

2.3. Patients and Healthy Controls Groups

Based on the 2010 ACR criteria [42], 12 patients (10 females and 2 males) suffering from the active form of RA, in the age range 18-65 years, referring to the Rheumatology Clinic of Loghman Hakim Hospital, Tehran, Iran, were selected and signed informed consent. The means of age and disease duration in them were 52.33 $\pm$ 1.65 and 8.08 $\pm$ 1.60 years, respectively. Although all the patients were being treated with conventional drugs including DMARDs (MTX 15-20mg/week, SSZ 500-1000mg/day and HCQ 400 mg/day), corticosteroids (PRD 5-15mg/day) and NSAIDs, for at least 6 months before this study, they had not shown adequate response to these drugs and their 28-joint Disease Activity Score (DAS28) was higher than 2.6 in all of them at baseline. The patients then consumed 1000mg/day of M2000 orally for 12 weeks. Furthermore, the physical examination and evaluation of the disease activity, as well as some labo-

ratory tests such as Erythrocyte Sedimentation Rate (ESR) and C - Reactive Protein (CRP), were done at the baseline and at the end of weeks 4 and 12. Moreover, the adverse effects of M2000 were checked every 2 weeks. Along with the patients, 12 healthy controls (10 females and 2 males) with age mean 43.75 $\pm$ 2.00 without any autoimmune and/or background diseases were selected in this study.

2.4. Sample Preparation

Venous blood specimens of the healthy controls and the qualified patients (before and after treatment with M2000) were collected in the heparinized venoject tubes. Peripheral Blood Mononuclear Cells (PBMCs) were then separated using Ficoll-Paque (Biosera Company, UAE) and after adding Trizol reagent (1mL for 10^7 cells) (Gene All Company, South Korea) were stoked at -70^{o}C.

2.5. RNA Extraction

Total RNA of 2×10^6 - 3×10^6 cells was extracted using the Hybrid-R™ Mini kit (Gene All Company, South Korea) based on the company's instructions and eluted in 48µl of nuclease-free water. Using NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific Company, USA), purity and concentration of extracted RNA were then evaluated. Afterward, in order to remove the genomic DNA (GDNA) contamination, treatment of the total RNA with RNase-free DNase I (Jena Bioscience Company, Russia) was accomplished based on the RNA concentration. The quantity and purity of DNase-treated RNA were re-evaluated using NanoDrop 2000 UV-Vis Spectrophotometer and in the next step, it was adjusted to the compactness 400ng in order to cDNA synthesis.

2.6. cDNA Synthesis and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

PrimerScript™ RT reagent Kit (Takara-Bio Company, Japan) with Random 6-mer and Oligo-dT primers, was used for cDNA synthesis. Based on the protocol of the Kit, the total volume of each cDNA synthesis reaction was 10µl containing 2µl Prime Script Buffer, 0.5µl Prime Script Reverse Transcriptase (RT) Enzyme Mix I, 0.5µl Oligo dT and 0.5µl Random 6-mer primers, dH2O and RNA with a concentration of 400ng. In order to reverse transcription, incubation was done in 37^{o}C for 15 minutes and RT inactivation was then accomplished through the incubation in 85^{o}C for 5 seconds.

The qRT-PCR reaction was performed by SYBR® Premix Ex Taq™ II (Takara-Bio Company, Japan), prepared cDNA and specifically designed primers for target genes (Bioneer Company South Korea) (Table **1**), based on the defined guidelines and using ABI StepOnePlus real-time PCR system (Applied Biosystems Company, USA). Each 20µl real-time PCR reaction included 1µl template cDNA, 10µl SYBR® Premix Ex Taq™ II, 0.4µl Rox, 0.8µl forward primer and 0.8µl reverse primer and 7µl nuclease-free water. In order to normalize the qRT-PCR reaction, Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) housekeeping gene was utilized as an internal control. The relative quantification of target genes mRNA was compared versus GAPDH gene mRNA and computed by the $2^{-\Delta\Delta Ct}$ method.

Table 1. Primer Pairs Sequences for Target Genes.

Genes	Primer Sequences
CXCR3	Fwd. TCTGCTGGACCCCCTATCAC
	Rev. CCACGTCTACCCTGCTTTCT
CXCR4	Fwd. ATCAGTCTGGACCGCTTCCT
	Rev. GACGCCAACATAGACCACCT
CCR2	Fwd. TACGGTGCTCCCTGTCATAAA
	Rev. TAAGATGAGGACGACCAGCAT
CCR5	Fwd. GCTCCCTACAACATTGTCCTTC
	Rev. GTCCAACCTGTTAGAGCTACTG
CCL2/MCP-1	Fwd. TCATAGCAGCCACCTTCATTC
	Rev. ACACTTGCTGCTGGTCATTC
GAPDH	Fwd. GAGAAGGCTGGGGCTCATTT
	Rev. TAAGCAGTTGGTGGTGCAGG

CCL2: C-C motif chemokine Ligand 2; CCR2: C-C chemokine Receptor type 2; CCR5: C-C chemokine Receptor type 5; CXCR3: C-X-C motif chemokine Receptor type 3; CXCR4: C-X-C motif chemokine Receptor type 4; Fwd: Forward primer; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase; MCP-1: Monocyte Chemoattractant Protein 1; Rev: Reverse primer.

2.7. Paraclinical Assessments

Enzyme-Linked Immunosorbent Assay (ELISA) (Euroimmun, Lübeck, Germany) was used for evaluating the serum level of Anti-Cyclic Citrullinated Peptide (Anti-CCP) antibodies and higher concentrations than 100IU/mL were considered as positive. The ESR as an essential factor for measuring the DAS28 index and evaluating the inflammatory reactions was assessed by the Westergren method, with considering the pathologic range of > 13 and > 20mm/hr for males and females, respectively. The Rheumatoid Factor (RF) and CRP levels were determined using a qualitative particle agglutination or latex test (Plasma-Tec Inc., USA). The positive agglutination tests for CRP and RF represented their serum levels about $\geq$ 1.0mg/dL and $\geq$ 15IU/mL, respectively.

2.8. Statistical Methods

Statistical analysis was carried out using a Statistical Package for the Social Sciences (SPSS) software (24.0; IBM Corporation, Armonk, NY, USA).

The normality or status of quantitative data dispersion was determined by the Shapiro-Wilk and Kolmogorov-Smirnov statistical tests. For the normally distributed data, Independent and Paired sample T-tests were used for between-group and intergroup (before and after treatment) comparisons, respectively. The Mann-Whitney U and Wilcoxon signed-rank statistical tests were utilized in order to assay similar comparisons, respectively, in connection with the non-normally distributed data. The quantitative variables have been exhibited as mean $\pm$ Standard deviation Error Mean (SEM).

Evaluation of qualitative data was done by Chi-square and McNemar statistical tests and they have been shown as numbers and percentages.

P-value ≤ 0.05 was considered as statistically significant. The statistical significance was classified as $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$.

3. RESULTS

3.1. Patients' Recovery

The 12-weeks oral consumption of the drug M2000 by the RA patients led to the reduction of their disease activity and pain. Moreover, improvement of the Modified Health Assessment Questionnaire-Disability Index (MHAQ-DI), ACR20 rate and clinical laboratory tests was observed in the patients after treatment with this new drug (Table **2**). This amelioration was observed even at the end of week 4. On the other hand, the anti-diabetic effect of M2000 was a considerable point, which was confirmed in some M2000-treated patients, in agreement with the previous report about this drug by Mortazavi-Jahromi *et al*. (2018) [44]. It should be noted that during the 12 weeks oral administration of M2000, no considerable adverse event was observed in the patients.

3.2. Effect of M2000 on mRNA Expression of CXCR4

The analysis of qRT-PCR results illustrated a higher CXCR4 mRNA expression in PBMCs of the patients before therapy with M2000 compared to the healthy controls (0.43-fold); however, this difference was not statistically significant ($P = 0.184$). Furthermore, following the oral administration of the M2000 by the patients for 12 weeks, a significant reduction was observed in the CXCR4 mRNA expression in PBMCs of the patients compared to the before treatment (1.16-fold with $P = 0.008$) (Fig. **1a**).

3.3. Effect of M2000 on mRNA Expression of CCR2

Based on the analyzed results of qRT-PCR, the CCR2 mRNA expression in PBMCs of the RA patients before treatment with M2000 was higher than the healthy controls (2.29-fold); however, the difference between them was not statistically significant ($P = 0.094$). Moreover, 12 weeks oral administration of the M2000 by RA patients significantly down-regulated the mRNA expression of CCR2 in PBMCs of the patients compared to the before treatment (2.20-fold with $P = 0.008$) (Fig. **1b**).

3.4. Effect of M2000 on mRNA Expression of CCL2/MCP-1

The analysis of qRT-PCR data demonstrated that CCL2/MCP-1 mRNA expression in PBMCs of the patients before therapy with M2000 was higher than the healthy controls (0.93-fold); however, this difference was not statistically significant ($P = 0.817$). In addition, following the oral administration of the M2000 by these patients for 12 weeks, a significant reduction was occurred in the mRNA expression of CCL2/MCP-1 in PBMCs of the patients compared to the before treatment (3.06-fold with $P = 0.041$) (Fig. **1c**).

3.5. Effect of M2000 on mRNA Expression of CXCR3 and CCR5

The analyzed findings of qRT-PCR represented that CXCR3 and CCR5 mRNAs expression in PBMCs of the patients before M2000 therapy was higher than the healthy controls (0.53- and 0.98-fold, respectively); however, their differences were not statistically significant ($P = 0.149$ and $P = 0.309$, respectively). Furthermore, although their mRNA expression reduced after 12 weeks oral administration of M2000 in these patients (1.55- and 1.59-fold, respectively), however, these reductions were not statistically significant ($P = 0.071$ and $P = 0.062$, respectively) (Fig. **1d** and **1e**, respectively).

4. DISCUSSION

The β-D-Mannuronic acid (M2000) as a new designed NSAID with approved anti-inflammatory and immunosuppressive properties is synthesized directly from Alginic acid by acidic hydrolysis method. The primary source of this drug is extensively used in the food industries and cosmetics. This new drug is almost/completely safe and without any prevalent adverse events, which are usually developed following the usage of various NSAIDs, including renal and gastrointestinal disorders, cardiopathy and heart failure. The previous studies demonstrated the potential therapeutic effects of M2000 in some animal models such as Adjuvant-Induced Arthritis (AIA), glomerulonephritis, nephrotic syndrome and Experimental Autoimmune Encephalomyelitis (EAE). Moreover, considerable tolerability and biocompatibility of M2000 compared to the other NSAIDs such as Diclofenac and Piroxicam and even steroids such as Dexamethasone have been confirmed [38, 41]. Preclinical assessment of the β-D-Mannuronic acid was performed, without any remarkable clinical and histopathological adverse events in chronic and sub-chronic toxicity evaluations by Fattahi *et al*. (2015). This investigation also showed that the oral administration of 1250 mg/kg of this drug, as the highest tested dose had no adverse effects [43]. Based on these findings, the amount of 25mg/kg/day has been considered as the optimized dose of M2000, which is almost/completely safe in humans. Regarding the substantial and remarkable outcomes of the previous studies, the Phase I/II clinical trials of this drug were distinctly designed on RA and Ankylosing Spondylitis (AS) patients on the years 2014-2016 and its safety, as well as efficacy, were evaluated in these patients [38, 45]. Recently, the randomized, placebo-controlled Phase III clinical trial of β-D-Mannuronic acid in RA patients has been accomplished as an international study and the safety, efficacy and therapeutic effects of this new drug have been confirmed in these patients leading to the reduction of disease activity [41]. The results of M2000 clinical trials were encouraging and demonstrated that the oral administration of this drug had no to low side effects and it was even able to modify the adverse effects of conventional drugs consumed simultaneously by patients [38, 41, 45]. The results of the present study showed that β-D-Mannuronic acid was able to down-regulate the gene expression of chemokine receptors and ligands, which can be led to the reduction of inflammatory reactions. The positive correlations between reduction in the

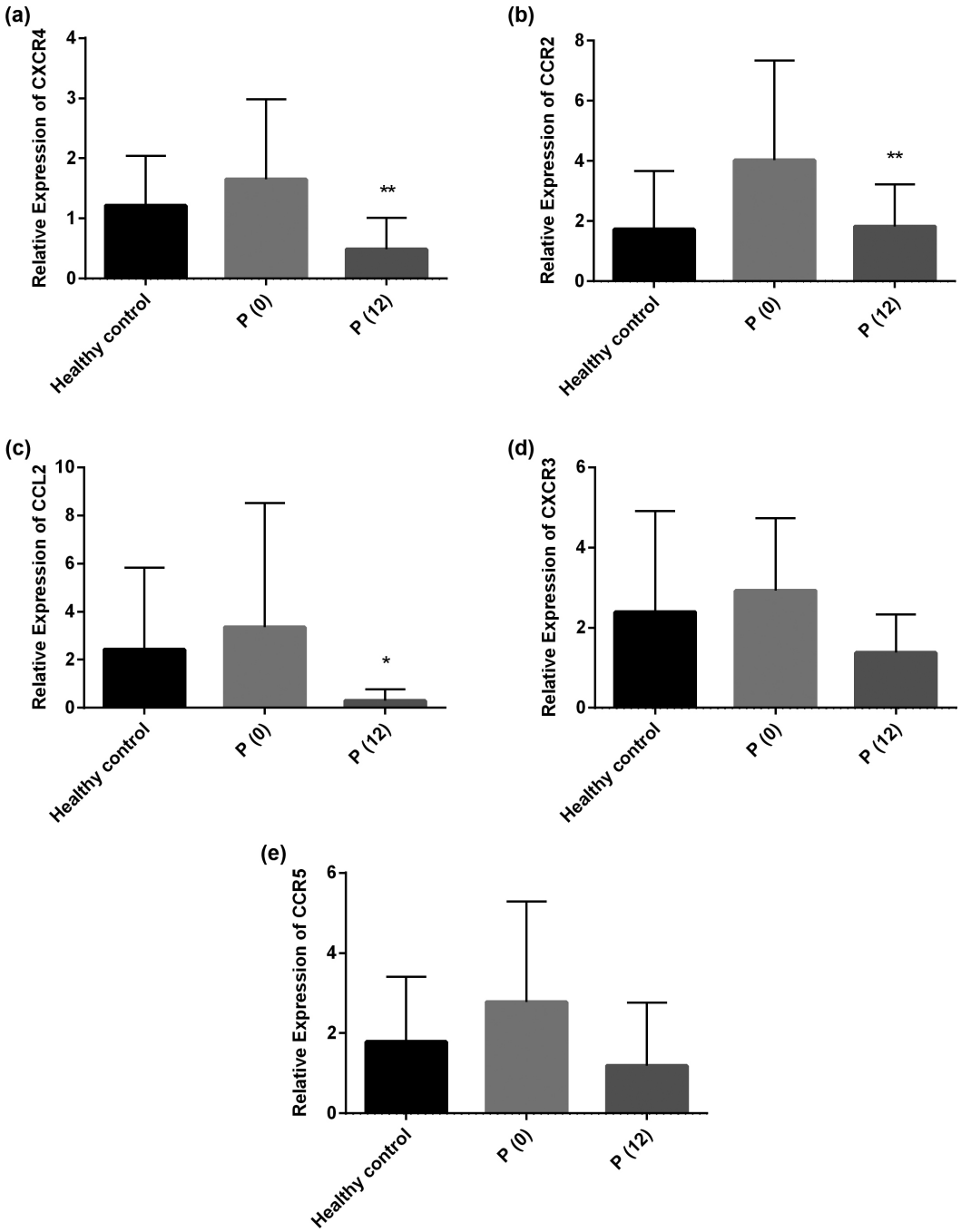

Fig. (1). Effect of M2000 oral administration on mRNA expression of CXCR4 (**a**), CCR2 (**b**), CCL2 (**c**), CXCR3 (**d**), and CCR5 (**e**). The relative quantifications of target genes were compared versus GAPDH gene and then calculated by the $2^{-\Delta\Delta Ct}$ method. The results have been represented as mean ± SEM. *P-value* ≤ 0.05 was considered as statistically significant. $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$ showed a significant reduction compared to the before treatment with M2000. HC: healthy control; P (0): patients before treatment with M2000; P (12): patients after 12 weeks treatment with M2000; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

gene expression of these parameters and clinical manifestations were also observed (Table **2**).

Along with our study, several investigations have been performed, which show the capability of DMARDs for reducing the chemokine receptors and ligands production. Ho *et al*. (2003) have reported that following the combination therapy of 15mg/week MTX and 10mg/day Leflunomide for 24 weeks in patients with RA, the mRNA expression of several chemokine ligands such as Thymus- and Activation-Regulated Chemokine (TARC), Macrophage-Derived Chemokine (MDC) as well as CCR2 and CCR4 chemokine receptors have been suppressed [46]. In addition, Ellingsen *et al*. (2007) assessed the oral administration effects of an adjusted dose of MTX (based on disease activity) during 12 weeks, on surface expression of CCR2 and CXCR3 in pe-

Table 2. Clinical and Paraclinical Improvement of RA Patients after 12 weeks M2000 Therapy.

Index	Before Treatment	After Treatment	*P Value*
Morning stiffness	41.25 ± 4.77	17.50 ± 5.62	0.008
Number of tender joints	4.00 ± 0.56	1.00 ± 0.27	0.001
Number of swollen joints	2.33 ± 0.64	0.50 ± 0.19	0.011
Patient assessment of pain	66.67 ± 3.95	40.00 ± 4.76	0.005
DAS28-ESR	4.46 ± 0.23	2.83 ± 0.11	0.001
DAS28 difference	-	-1.63 ± 0.21	-
ACR20	6.58 ± 0.52	5.25 ± 0.35	0.047
MHAQ-DI	0.96 ± 0.19	0.22 ± 0.11	0.006
PGA	100 ± 0	27.50 ± 4.94	0.001
ESR	22.33 ± 3.83	14.08 ± 2.65	0.016
Anti-CCP	207.75 ± 83.43	201.13 ± 80.71	0.068
RF	58.3% (Positive)	50.0% (Positive)	1.000
CRP	41.7% (Positive)	33.3% (Positive)	1.000

ACR20: American College of Rheumatology 20; Anti-CCP: Anti-Cyclic Citrullinated Peptide; CRP: C - Reactive Protein; DAS28: 28-joint Disease Activity Score; DAS28 difference: Difference of DAS28 after and before M2000 therapy; ESR: Erythrocyte Sedimentation Rate; MHAQ-DI: Modified Health Assessment Questionnaire-Disability Index; PGA: Patient Global Assessment; RF: Rheumatoid Factor.

ripheral circulating monocytes and $CD4^+$ T lymphocytes of the patients with the active form of RA. They have shown that this drug can decline the CCR2 expression in monocytes, while the expression of CCR2 in T lymphocytes and CXCR3 in both types of the cells has not been affected by MTX [47]. On the other side, numerous studies have demonstrated the inhibitory effects of NSAIDs on chemokine receptors and ligands. Liang *et al*. (2003) illustrated that followed by daily oral administration of 50mg/kg of Celecoxib for 15 days, the mRNA expression of CXCR4, CCR2, CCR5 chemokine receptors and CCL2/MCP-1 chemokine ligand has reduced significantly in irradiated skin tissue [48]. The results of our study were in agreement with these findings. 12-weeks β -D-Mannuronic acid-treatment could significantly down-regulate the mRNA expression of CXCR4, CCR2 and CCL2/MCP-1 (Fig. **1a**, **1b**, and **1c**, respectively), and their reduction had a positive association with disease activity without any adverse events. It should be noted that the reduction of CXCR3 and CCR5 mRNAs expression was also near to a significant level (Fig. **1d**, and **1e**, respectively). It has been reported that the CXCR4/ CXCL12 interaction has a critical role in the migration and survival of inflammatory cells into the synovium [12, 49, 50]. The CCR2/ CCL2 binding has also a fundamental role in synovial angiogenesis and bone erosion [12, 13]. Therefore, as these molecules play a crucial role in RA progression and inflammatory reactions, their declining can be an appropriate therapeutic strategy.

Our findings in the present study show that M2000 is probably able to perform a similar role of DMARDs and other NSAIDs in restricting the infiltration of inflammatory cells into the synovium, through the reduction of the chemokine receptors and ligands expression.

CONCLUSION

Collectively, our data demonstrate the anti-inflammatory and immunosuppressive properties of β-D-Mannuronic acid more than before. Comparing the results of the present research and the above mentioned investigations in connection with DMARDs and NSAIDs shows that M2000 similar to these drugs can down-regulate the gene expression of chemokine receptors and ligands such as CXCR4, CCR2 as well as CCL2/MCP-1, and probably restrict the recruitment and infiltration of the inflammatory cells into the synovium, which in turn leads to the reduction of inflammation. Therefore, this novel drug might be recommended for ameliorating the quality of life in patients with RA and be efficient in the treatment of this disease.

CURRENT & FUTURE DEVELOPMENTS

Based on our current *in vitro* and *in vivo* studies, particularly the Phase III clinical trial of drug M2000 on patients with RA, the therapeutic effects and safety of this drug have been confirmed in these patients. On the other hand, the results of our investigations on some other autoimmune disorders in different experimental and animal levels, and in some cases in Phase I/II clinical trials have confirmed the anti-inflammatory and immunosuppressive properties of the drug M2000, accompanied by its therapeutic efficacy. Accordingly, running several Phase I, II, and III clinical trials for evaluation of this drug efficacy and safety in other inflammatory-autoimmune diseases is among our plans.

LIST OF ABBREVIATIONS

^{13}C-NMR = Carbon-13 Nuclear Magnetic Resonance

ACR = American College of Rheumatology

AIA = Adjuvant-Induced Arthritis
Anti-CCP = Anti-Cyclic Citrullinated Peptide
AS = Ankylosing Spondylitis
CCL2 = C-C Motif Chemokine Ligand 2
CCR2 = C-C Chemokine Receptor Type 2
CCR5 = C-C Chemokine Receptor Type 5
CRP = C - Reactive Protein
CXCL9 = C-X-C Motif Chemokine Ligand 9
CXCL10 = C-X-C Motif Chemokine Ligand 10
CXCL12 = C-X-C Motif Chemokine Ligand 12
CXCR3 = C-X-C Motif Chemokine Receptor Type 3
CXCR4 = C-X-C Motif Chemokine Receptor Type 4
DAS28 = 28-Joint Disease Activity Score
DCs = Dendritic Cells
DMARDs = Disease-Modifying Anti-Rheumatic Drugs
EAE = Experimental Autoimmune Encephalomyelitis
ELISA = Enzyme-Linked Immunosorbent Assay
ESR = Erythrocyte Sedimentation Rate
FTIR = Fourier Transform Infrared
GAPDH = Glyceraldehyde-3-Phosphate Dehydrogenase
GDNA = Genomic DNA
HCQ = Hydroxychloroquine
IP-10 = Interferon-Inducible Protein 10
M2000 = β-D-Mannuronic Acid
MCP-1 = Monocyte Chemoattractant Protein 1
MDC = Macrophage-Derived Chemokine
MHAQ-DI = Modified Health Assessment Questionnaire-Disability Index
MIG = Monokine Induced by Gamma Interferon
MTX = Methotrexate
NSAIDs = Non-Steroidal Anti-Inflammatory Drugs
PB = Peripheral Blood
PBMCs = Peripheral Blood Mononuclear Cells
PRD = Prednisolone
qRT-PCR = Quantitative Real-time Polymerase Chain Reaction
RA = Rheumatoid Arthritis
RF = Rheumatoid Factor
RT = Reverse Transcriptase
SEM = Standard Deviation Error Mean
SF = Synovial Fluid
SPSS = Statistical Package for the Social Sciences
SSZ = Sulfasalazine
STs = Synovial Tissues
SDF-1 = Stromal Cell-Derived Factor 1
TARC = Thymus- and Activation-regulated Chemokine

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This investigation was accepted by the Ethics Committee of Mashhad University of Medical Sciences (MUMS) (No.IR.MUMS.fm.REC.1396.309, Mashhad, Iran) and trial registration number IRCT2017100213739N10 was then obtained. Written informed consent was signed by all the enrolled patients and healthy controls.

HUMAN AND ANIMAL RIGHTS

No animals were used in this research. The study was conducted based on American College of Rheumatology (ACR) criteria [42] and Helsinki manifest guidelines.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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