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ORIGINAL ARTICLE

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The genes expression status of inflammatory determinants following the oral administration of Mannuronic acid in patients with rheumatoid arthritis

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ABSTRACT

Introduction: Rheumatoid arthritis (RA) is a progressive multifactorial inflammatory disorder. According to numerous evidence, pro-inflammatory markers such as TNF-α, IL-6, IL-22, MYD88 and TLR2 play a substantial role in the pathogenesis and persistence of this disease. B-D-Mannuronic acid (M2000) is a new immunosuppressive drug whose therapeutic effects have been approved in several clinical trials and the results of the phase III clinical trial of this drug in RA patients were potent and efficient. Therefore, the present investigation was designed to evaluate its anti-inflammatory effects on the expression of mentioned factors in RA patients.

Material and methods: This research was carried out on 12 healthy individuals and 12 patients with RA and M2000 was administered to the patients orally at a dose of 500 mg twice daily for 12 weeks. The peripheral blood mononuclear cells (PBMCs) were collected from the patients before and after treatment with M2000 to investigate the gene expression levels of *TNF-α, IL-6, IL-22, MYD88* and *TLR2* molecules in them using Real-time PCR.

Results: This study data represented a higher gene expression in all target molecules in the RA patients in comparison to the healthy individuals. Furthermore, the outcomes showed that after 12 weeks of therapy with M2000, the gene expression levels of inflammatory factors TNF-α, IL-6, IL-22, MYD88 and TLR2 decreased significantly in treated patients compared to before therapy. The gene expression results were following the clinical and paraclinical assessments.

Conclusion: In conclusion, M2000 as a newly approved anti-inflammatory and immunosuppressive drug, can be proposed as a therapeutic agent in RA patients.

Key words: mannuronic acid, M2000, rheumatoid arthritis, TNF-α, IL-6, IL-22, MYD88, TLR2

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Introduction

Rheumatoid arthritis (RA) is the most common autoimmune and inflammatory disease, which is characterized by symmetrical inflammation of the synovium and variable degrees of bone and cartilage destruction that cause systemic manifestations in various organs and reduced mobility. RA has also a significant negative effect on the ability and life quality of patients [1].

The exact aetiology of RA is unknown yet. Many documents represented the crucial role of complex interactions between genetic factors such as human leukocyte antigen-DRB1 (HLA-DRB1), Protein tyrosine phosphatase, non-receptor type 22 (PTNP22), Cytotoxic

T-Lymphocyte Associated Protein 4 (CTLA4), Cluster of Differentiation 28 (CD28), TNF receptor-associated factor 1 (TRAF1) and Signal transducer and activator of transcription 4 (STAT4) and environmental factors like smoking, gender- and age-associated factors in the incidence of this disease [2, 3]. It is approximated that 0.5–1% of the world's population with a female: male ratio of 3:1 is RA patients. Rheumatoid factor (RF) and anti-citrullinated peptide antibodies (Anti-CCPs) are clinical determinants that their production can precede even several years before disease onset in RA patients. C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) are also the two inflammatory indices applied for the disease-activity assessment. Although a diagnosis of RA is based upon clinical manifestations, particularly joint characteristics (counts of tender and swollen joints), however, the presence of RF and Anti-CCP in serum is more specific for the diagnosis of this disease. American College of Rheumatology (ACR) response rates are used to define clinical response in clinical trials [4, 5]**.** Pro-inflammatory cytokines, innate immunity receptors and their adaptor proteins have great roles in the induction of autoimmune mechanisms and chronic infiammation involved in the pathogenesis of RA. Among them, Tumour necrosis factor α (TNF-α), interleukin 6 (IL-6), IL-22, Toll-like receptor 2 **(**TLR2) and Myeloid differentiation primary response 88 (MYD88) play an indispensable role in the immunopathogenesis of RA and are considered as therapeutic targets in this disease.

TNF-α as a basic cytokine in the intensifying of RA motives inflammatory and tissue-resident cells like B and T lymphocytes, monocytes, macrophages, fibroblasts and osteoclasts towards the expression of inflammatory mediators like cytokines, chemokines, proteases, anti-apoptotic and angiogenic factors, adhesion molecules as well as osteoclastogenic mediators. The differentiation of Th22 and Th17 lymphocytes as well as M1 macrophages are implemented via TNF-α [6, 7]. Substantially, TNF-α is recently considered the first target in the treatment of patients with RA [7, 8]. TNF-α inhibition results in the attenuation of symptoms and reduction of disease activity in the patients [9]. IL-6 as the second crucial cytokine involved in the pathogenesis of RA plays analogous roles with TNF-α in the pathogenicity of this disease. IL-6 stimulates hepatic cells for synthesizing the acute phase reactants [CRP, Serum amyloid A (SAA)] and has also an important role in anaemia of chronic disease *(*ACD) [10]. As high levels of IL-6 have been observed in autoimmune diseases such as RA, it prospects that its inhibition can be effective in the improvement of patients with RA. This cytokine stimulates nuclear factor kappa B (NF-KB) and STAT/RAS/MAP/AKT pathways, therefore, it has a significant role in the expression of pro-inflammatory, angiogenic and osteoclastogenic factors, antibody production, differentiation and amplification of immune cells like B and T lymphocytes and macrophages, as well as osteoblasts differentiation inhibition. IL-6 levels are also associated with clinical characterization and joint erosion [11–13].

Another key cytokine in RA pathogenesis is IL-22 which induces the expression of inflammatory mediators, osteoclastogenic and anti-apoptotic factors as well as proteases. This factor is produced by various cells such as macrophages, Th17 and Th22 as well as fibroblast cells in RA [14, 15]. It has been shown that high amounts of IL-22 are expressed in peripheral blood (PB) and synovial fluid (SF) of a patient with RA which is associated with Th17 and Th22 lymphocytes frequency and clinical parameters, such as Disease Activity Score-28 (DAS28) as an index of disease activity, RF and Anti-CCP**.** Suppression of IL-22 is a therapeutic approach in RA patients [16]. TLR2 is a molecule whose overexpression in immune cells like monocytes, macrophages and T cells leads to the severity of inflammation and joint destruction in RA. Macrophages in RA patients are more sensitive and responsive to TLR2 ligands compared to the macrophages of healthy controls. This receptor with attachment to pathogen-associated molecular patterns (PAMPs), such as proteoglycans, lipoproteins, peptidoglycan, lipoteichoic acid, etc) and damage-associated molecular patterns (DAMPs) such as snapin, SAA and heat shock proteins (HSPs) that are expressed in high levels in PB, SF and synovial tissues of RA patients, exacerbates disease progression. TLR2 as an innate immunity receptor implicates in the pathogenesis of RA, via provocation of inflammatory pathways such as NF-KB, MAPK, AP-1, P38/ERK, JAK/STAT, mTOR/AKT/PI3K, which their products, including cytokines, MMPs and costimulatory adhesion factors have an essential role in disease persistence. On the other hand, TLR2 participates in the differentiation of Th17 and Th9 cells as well as M1 macrophages. MYD88 as one of the vital adaptor proteins in TLRs signal transduction and NF-KB provocation has an essential role in the intensification of inflammation and disease activity in RA [17–22].

There are five classes of drugs, which are currently used in the treatment of patients with RA including disease-modifying anti-rheumatic drugs (DMARDs), biologic response modifiers (a type of DMARDs), non-steroidal anti-inflammatory drugs (NSAIDs), analgesics and corticosteroids. NSAIDs have a short onset of efficacy but DMARDs and corticosteroids have a long time and notable efficacy during several weeks or months [23]. Moreover, DMARDs are considered selective drugs for reducing the disease activity, nevertheless, due to the lack of adequate response of patients to these

drugs and their serious side effects in various systems, such as the gastrointestinal tract, kidney, liver, as well as increasing Hb level, toxicity, latent infections and muscles weakness, their therapeutic value has been limited. Therefore, a new therapy is required for those RA patients who had an inadequate response to conventional therapy and it can be effective in the treatment of these patients [24].

The β-D-Mannuronic Acid (M2000) patented (DE-102016113018) with low molecular weight (194.139Da) is a new NSAID and one of the alginic acid comonomers that has extensive anti-infiammatory and immunosuppressive properties as well as therapeutic effects. The anti-inflammatory effects and also safety of this drug have been documented in various animal models like adjuvant-induced arthritis (AIA), Experimental *autoimmune* encephalomyelitis (EAE), nephrotic syndrome and acute gleumeluronephritis as well as variant clinical trials. It should be noted that the oral administration of the β-D-Mannuronic acid in the international phase III clinical trial of this drug in patients with active RA has significantly demonstrated its safety and efficacy on DAS28, morning stiffness and counts of tender and swollen joints [25–29].

According to the above-mentioned data, in the present investigation, the efficacy of M2000 on gene expression of TNF-α, IL-6, IL-22, MYD88 and TLR2 molecules was evaluated in RA patients with the active form of the disease that had an inadequate response to conventional therapies. In the end, the results were compared to the healthy controls.

Material and methods

Ethical approval

The study was confirmed by the ethics committee of Mashhad University of Medical Sciences (MUMS) (NO.IRIR.MUMS.fm.REC.1396.309) and trial registration (IRCT2017100213739N10) for this new drug was obtained. This research was done under principles established by the ACR and Helsinki guidelines. Written informed consent also was taken from all the patients.

Production of M2000

A small molecule of M2000 with the chemical formula ($C_6H_{10}O_7$) and molecular weight 194.139 Da was synthesized from sodium alginate (Sigma-Aldrich, St Louis, MO, USA) according to pf Mirshafiey et al, method [29]. Afterwards, the purity of M2000 was checked by characterizing the hydrolytic products using Fourier transform infrared (FTIR) spectroscopy and carbon-13 nuclear magnetic resonance (C-NMR) spectroscopy.

Study subjects and oral administration of M2000

This investigation was performed on 12 RA patients with age 18–65 years. They were selected based on ACR20 criteria in an active phase of the disease with resistance to prevalent therapies, to 12 weeks of M2000 therapy. The patients were chosen from the Rheumatology Departments of Loghman Hakim Hospital (Tehran, Iran).

The mean age in the selected patients (females 10, males 2) was 52.33 ± 1.65 years and the mean disease duration was 8.08 ± 1.60 years. M2000 therapy in these patients was done on Oct. 4, 2017, to Oct. 8, 2018. These patients were assigned based on having ≥ 6 tender joints and 3 and/or more than 3 (≥ 3) swollen joints in 28 joints. Moreover, the levels of ESR (ELISA), Anti-CCP (ELIZA) and CRP (Latex agglutination method) were more than the normal range and also the duration of morning stiffness was ≥ 30 min. The patients were receiving DMARDs + corticosteroids + NSAIDs at baseline. They were receiving a dose of 15–20 mg/week of Methotrexate (MTX), Sulfasalazine (SSZ) 500–1000 mg/day, Hydroxychloroquine (HCQ) 400 mg/day, Prednisolone (PRD) 5–15 mg/day, and NSAIDs before this clinical trial.

Based on the preclinical evaluations, a minimum dosage (18 mg/kg/d) of M2000 (500 mg) was prescribed twice daily for 12 weeks. Probable adverse events of M2000 were checked, regularly. Besides, the clinical efficacy and safety of this drug in the patients were evaluated by the ACR20 response. Thereafter, the patients were visited at the baseline and 4 and 12 weeks after M2000 therapy. During 12 weeks of the trial, the patients consumed their conventional medications. Furthermore, there were 12 healthy donors as a control group (10 females, 2 males) with a mean of age 43.75 ± 2.00 and a lack of autoimmune and infectious disorders.

Sample preparation/RNA extraction

Blood samples were taken from normal controls and RA patients (before and after treatment with M2000). Afterwards, PBMCs were separated using Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) and stored at –70°C. The total RNA was then extracted from the cells by a total RNA purification kit (Hybrid R Gene All, Seoul, Korea) based on the manufacturer's protocol. The concentration of extracted RNA was measured using NanoDrop 2000 UV–Vis Spectrophotometer (Isogen Life Science, Netherlands). In the next step, RNA was treated with RNase-free DNase I (Jena Bioscience, Germany) to eliminate the genomic DNA. The concentration of RNA was assessed by nanodrop (Isogen Life Science, Netherlands).

cDNA synthesis and quantitative real-time polymerase chain reaction (qRT-PCR)

cDNA was synthesized from the total RNA with a concentration of 400 ng based on an instruction of the cDNA synthesis kit (Takara Co., Ltd., Dalian, JAPAN). Both oligo-dt and random hexamer primers were utilized for reverse transcription

RT-PCR was fulfilled using SYBER PREMIX EX TAG II (TAKARA, JAPAN), synthesized cDNA and specific primers (BIONEER, KOREA) (Tab. 1), in ABI STEP ONE PLUS Real-Time PCR system (ABI, USA).

Table 1. Specific primers for target genes in RT-PCR

Primer sequences	Gene
F: CACAGTGAAGTGCTGGCAAC *R: AGGAAGGCCTAAGGTCCACT*	*TNF-a*
F: ACTCACCTCTTCAGAACGAATTG *R: CCATCTTTGGAAGGTTCAGGTTG*	*IL-6*
F: GCTTGACAAGTCCAACTTCCA *R: GCTCACTCATACTGACTCCGT*	*IL-22*
F: GAATCCTCCAATCAGGCTTCTCT *R: GCCCTGAGGGAATGGAGTTTA*	*TLR2*
F: ATGAAGAAAGAGTTCCCCAGCA *R: CAAGGCGAGTCCAGAACCAAG*	*MYD88*
F: GAGAAGGCTGGGGCTCATTT *R:TAAGCAGTTGGTGGTGGTGGTGCAGG*	*GAPDH*

F — forward; R — reverse; TNF-α — *tumor necrosis factor* α; IL-6 — Interleukin 6; IL-22 — Interleukin 22; TLR2 — Toll like receptor 2; MYD88 — Myeloid differentiation primary response 88; GAPDH — Glyceraldehyde-3-Phosphate Dehydrogenase

The Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) as an internal control gene was also used for normalizing the amplification. The relative amounts of PCR products were measured through the $2^{-\Delta\delta Ct}$ method.

Statistical analysis

The statistical analysis was executed using *Statistical Package for the Social Sciences* (SPSS) software (24.0; IBM Corporation, Armonk, NY, USA). The data were compared by paired samples T-test and Wilcoxon test to determine significant differences in the gene expression, before and after treatment with M2000. And by Mann–Whitney U test and Independent sample T-test to the comparison between healthy controls and patients, before treatment with M2000. The data were represented as mean ± SEM and a P-value ≤ 0.05 was considered statistically significant. Quantitative data were evaluated using chi-square and Mc Nemar tests.

Results

Patient's response

The patients recovered after 4 weeks of therapy and their improvement also continued during the treatment course. Clinical and laboratory parameters including DAS28, ESR, counts of tender and swollen joints, morning stiffness, patient assessment of pain, Patient global assessment, ACR and *Modified Health Assessment Questionnaire* (MHAQ) had a significant reduction after 12 weeks of M2000 therapy (Tab. 2).

Table 2. The clinical and laboratory findings of the RA patients before and after treatment with M2000

Index	Before treatment (n = 12)	After treatment (n = 12)	P-value
DAS28 (0–10)/activity score	4.46 ± 0.23	2.83 ± 0.11	0.001
DAS28 Difference	–	–1.63 ± 0.21	–
ACR	6.58 ± 0.52	5.25 ± 0.35	0.047
Tender joints count (number)	4.00 ± 0.56	1.00 ± 0.27	0.001
Morning Stiffness (quality)	41.25 ± 4.77	17.50 ± 5.62	0.008
Swollen joints count (number)	2.33 ± 0.64	0.50 ± 0.19	0.011
Patient assessment of pain	66.67 ± 3.95	40.00 ± 4.76	0.005
Patient global assessment (0–100)/(disease activity)	100 ± 0	27.50 ± 4.94	0.001
MHAQ	0.96875 ± 0.19	0.22917 ± 0.11	0.006
ESR (millimetres/mm per hour)	22.33 ± 3.83	14.08 ± 2.65	0.016
CRP (mg/L)	41.7% (Positive)	33.3% (Positive)	1.000
RF (IU/mL)	58.3% (Positive)	50% (Positive)	1.000
Anti-CCP (IU/mL)	207.75 ± 83.43	201.13 ± 80.71	0.068

ACR — American College of Rheumatology; Anti-CCP — anti-citrullinated peptide antibody; *CRP — C-reactive protein;* DAS28 — disease activity score 28 joints; ESR — *erythrocyte sedimentation rate;* MHAQ — *Modified Health Assessment Questionnaire;* RF — rheumatoid factor. DAS28 differentiation: The difference in DAS28 amount at the baseline and after 12 weeks of M2000 therapy

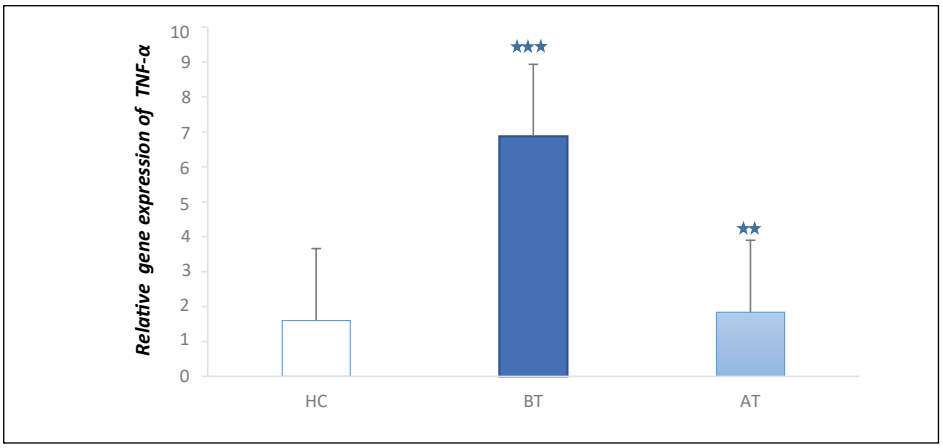

Figure 1. Comparison of TNF-α gene expression between the studied groups. The statistical significance was classified as **P ≤ 0.01, *** P ≤ 0.001. AT — after treatment with M2000; BT — before treatment with M2000; HC — healthy control

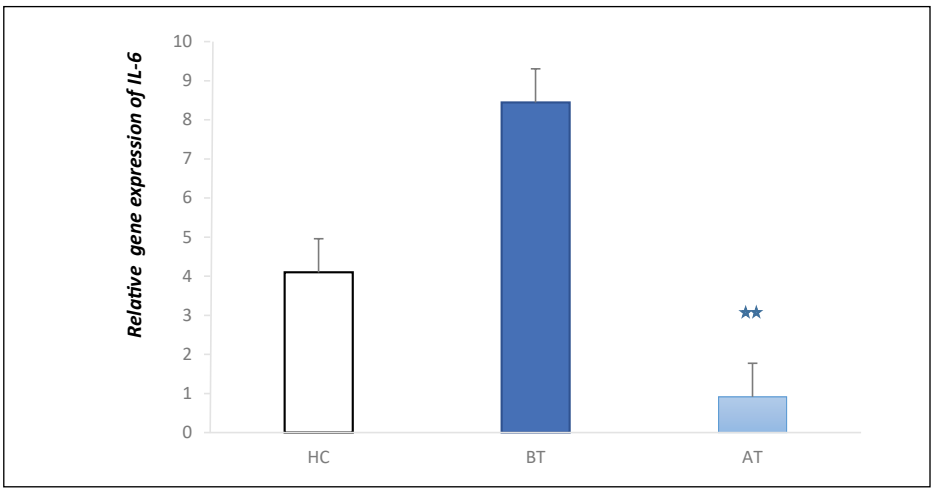

Figure 2. Comparison of IL-6 gene expression between the studied groups. The statistical significance was classified as **P ≤ 0.01. AT — after treatment with M2000; BT — before treatment with M2000; HC — healthy control

Effects of M2000 on TNF-α gene expression

The present findings demonstrated that TNF-α gene expression was significantly higher in the RA patients compared to the healthy controls (a 5.35-fold reduction was found, p-value ≤ 0.001).

On the other hand, the data showed that after 12 weeks of therapy with M2000, *TNF-α* gene expression in the patients decreased significantly, in comparison to the before therapy (a 5.10-fold reduction, P-value ≤ 0.01 (Fig. 1).

Effects of M2000 on IL-6 gene expression

This study results represented a higher gene expression of IL-6 in the RA patients compared to the healthy controls, however, this difference was, not significant, statistically (a 4.34-fold reduction, p-value > 0.05).

On the other hand, the data showed that after 12 weeks of therapy with M2000, IL-6 gene expression in the patients declined significantly, in comparison to the before therapy (a 7.53-fold reduction, p-value < 0.01) (Fig. 2).

Effects of M2000 on MYD88 gene expression

The present findings indicated a significantly higher gene expression of MYD88 in the RA patients in comparison to the healthy controls (a 0.36-fold reduction, p-value < 0.05).

On the other hand, the data showed that after 12 weeks of therapy with M2000, MYD88 gene expression in the patients decreased significantly in comparison to the before therapy (a 0.81-fold reduction, p-value = 0.01) (Fig. 3).

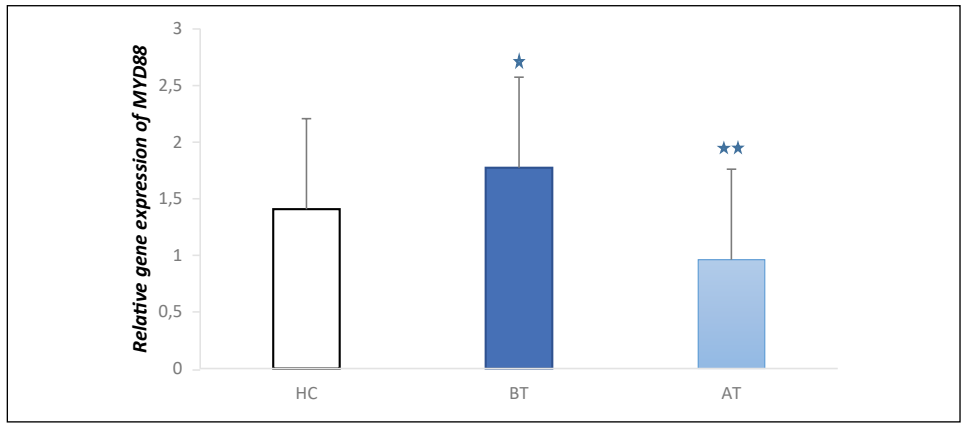

Figure 3. Comparison of MYD88 gene expression between evaluated the studied groups. The statistical significance was classified as *P ≤ 0.05, **P ≤ 0.01. AT — After treatment with M2000; BT — Before treatment with M2000; HC — Healthy control

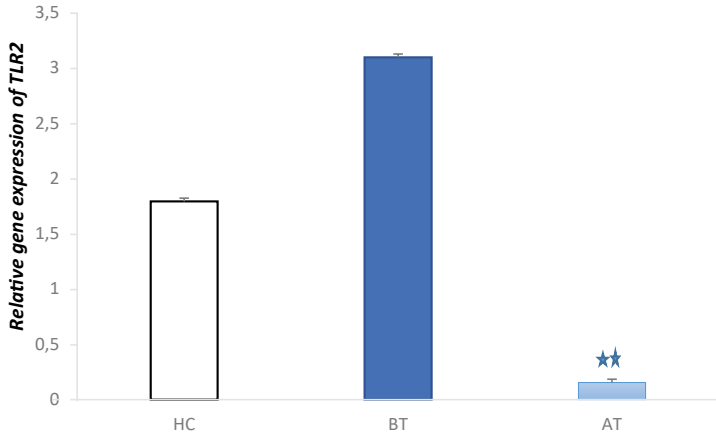

Figure 4. Comparison of TLR2 gene expression between the studied groups. The statistical significance was classified as **P ≤ 0.01. AT — After treatment with M2000; BT — Before treatment with M2000; HC — Healthy control

Effects of M2000 on TLR2 gene expression

This study's findings demonstrated that TLR2 gene expression was higher in the RA patients compared to the healthy controls, however, this difference wasn't significant (a 1.30-fold reduction, p-value > 0.05). On the other hand, the data showed that after 12 weeks of therapy with M2000, TLR2 gene expression in the patients declined significantly, compared to the before therapy (a 2.94-fold reduction, p-value < 0.01) (Fig. 4).

Effects of M2000 on IL-22 gene expression

The outcomes indicated that *IL-22* gene expression was higher in the RA patients compared to the healthy controls, however, this difference wasn't significant, statistically (a 2.27-fold reduction, p-value > 0.05).

On the other hand, the data showed that after 12 weeks of therapy with M2000, *IL-22* gene expressions in the patients reduced significantly, in comparison to the before therapy (a 4.80-fold reduction, p-value < 0.01) (Fig. 5) (Tab. 3).

Discussion

The inflammatory milieu in the RA disease is regulated by the complex cytokines and TLRs network. Clinical interventions supported the role of molecules TNF-α, IL-6, IL-22, TLR2 and MYD88 in this process. Above mentioned markers cause the induction or aggravation of the inflammatory reactions by agitating the inflammatory cells and infiltrating them to accumulate within the synovial compartments [30]. Inflammation is the crucial

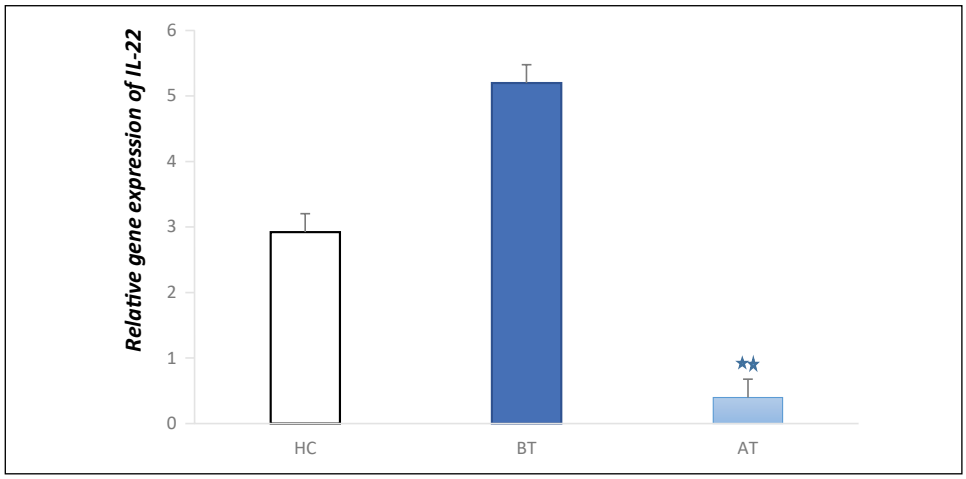

Figure 5. Comparison of IL-22 gene expression between the studied groups. The statistical significance was classified as **P ≤ 0.01. AT — After treatment with M2000; BT — Before treatment with M2000; HC — Healthy control

Table 3. The results of the gene expression of inflammatory factors in RA patients, before and after treatment with M2000

Index	Healthy control/before treatment (n = 12) (n = 12)/fold change	Before treatment/after treatment (n = 12)/fold change	Healthy control/before treatment (n = 12)/p-value	Before treatment/after treatment (n = 12)/p-value
TNF-α	5.35	5.10	≤ 0.001	≤ 0.01
IL-6	4.34	7.53	≥ 0.05	≤ 0.01
MYD88	0.36	0.81	≤ 0.05	0.01
TLR2	1.30	2.94	≥ 0.05	≤ 0.01
IL-22	2.27	4.80	≥ 0.05	≤ 0.01

point in clinical events (driving clinical symptoms, joint damage, disability and co-morbidity), therefore, it is considered the major therapeutic target. If inflammation attenuates rapidly, the disease progression will be prevented and physical function will improve**.** Collectively, TLR2 can be regarded as a suitable therapeutic target. Dysregulation of cytokines and TLRs network in RA disease can promote inflammation not only in joints but also in other organs and tissues of the body [31]. Generally, DMARDs are used to diminish disease activity in RA patients [32, 33], however, some patients do not respond to these treatments and show inadequate response to them. Moreover, RA patients are suffering from the adverse effects of these drugs [34–36].

In line with the present study, diverse investigators have reported the below results. Barcelos et al. studied the efficacy of Diclofenac on the TLR4-nuclear factor kappa light chain (NF-κB) signalling pathway for the diminishment of exercise-related inflammation. The results of this study indicated that this drug could significantly reduce the production of the TNF-α, IL-6, and MYD88 adaptor protein [37]. In a study, Huang et al. measured the IL-6 serum level in the Parecoxib-treated group which had kidney disease. Following the treatment with this NSAIDs, the serum level of this cytokine decreased, significantly [38]. Grosky and coworkers evaluated the effects of the Lornoxicam on TLR2 mRNA expression as well as serum levels of the TNF-α and IL-6 in patients with acute pancreatitis. The data represented a significant decrease in TLR2 mRNA expression and production of the pro-inflammatory cytokines, followed by the reduction of systemic complications and mortality [39].

M2000 as a novel NSAID with immunosuppressive properties has shown potent therapeutic effects, safety and notable efficacy on multiple experimental animal models. Furthermore, its anti-inflammatory and immunosuppressive properties as well as the therapeutic efficacy of this drug have been demonstrated in several clinical trials [28]. The efficacies of this drug as well as its great tolerability and biocompatibility have been documented in various studies. Furthermore, the therapeutic effects of M2000 have been confirmed in clinical trials I/II Ankylosing spondylitis (AS) and RA. Moreover, a phase III clinical trial of this novel drug in RA patients has been accomplished, and its results were approving M2000 efficacy on a large scale [28]. Regarding the importance of NF-κB instigation in the

expression of inflammatory mediators such as TNF-α, IL-6 and based on the key role of TLR2 in triggering this transcription factor, targeting this factor can be very effective in the reduction of inflammation and improvement of the patients. It has been approved that, M2000 as a new NSAID, could reduce the expression of TLR2, MYD88, TRAF-6, Interleukin 1 Receptor Associated Kinase 1 (IRAK1) and NF-κB, as well as TNF-α and IL-6. Therefore, M2000 can be propounded as a new therapeutic approach [40–55]. In this study, a significant correlation between the inflammatory gene expression results and clinical as well as laboratory findings was observed. The levels of these markers were in accordance with the disease activity (DAS28), tender joint and swelling, which had a reduction in mean after treatment with M2000. In the present study after 12 weeks of M2000 therapy a potent inhibitory effect of this drug was observed on the expression of inflammatory markers in RA patients. This novel drug showed anti-inflammatory and great tolerability during the treatment course and its consumption had no serious adverse effects. This clinical trial revealed the potent anti-inflammatory and immunosuppressive properties of M2000 in 12 RA patients based on the status of the pro-inflammatory cytokines profile.

Conclusion

Based on the clinical assessments and gene expression results, it can be concluded that the oral administration of M2000 as a natural novel NSAID with immunosuppressive properties, may help the improvement of RA patients that had an inadequate response to conventional therapies. Therefore, it is suggested that M2000 therapy can be used as an effective therapy for patients with RA and probably other autoimmune diseases.

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