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ORIGINAL RESEARCH

# Effects of β-D-mannuronic acid, as a novel non-steroidal anti-inflammatory medication within immunosuppressive properties, on *IL17*, *RORγt*, *IL4* and *GATA3* gene expressions in rheumatoid arthritis patients



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**Abstract:** Rheumatoid arthritis (RA) is the most common form of chronic inflammatory arthritis characterized by pain, swelling and destruction of joints, with a resultant disability. Disease-modifying anti-rheumatic drugs (DMARDs) and biological drugs can interfere with the disease process. In this study, the effect of β-D-mannuronic acid (M2000) as a novel non-steroidal anti-inflammatory drug (NSAID) with immunosuppressive and anti-inflammatory effects together with antioxidant effects was evaluated on *IL17*, *RORγt*, *IL4* and *GATA3* gene expression in 12 RA patients. Previously, M2000 driven from sodium alginate (natural product; patented, DEU: 102016113018.4) has shown a notable efficacy in experimental models of multiple sclerosis, RA and nephrotic syndrome. This study was performed on 12 patients with RA who had an inadequate response to conventional treatments. During this trial, patients were permitted to continue the conventional therapy excluding NSAIDs. M2000 was administered orally at a dose of 500 mg twice daily for 12 weeks. The peripheral blood mononuclear cells (PBMCs) were collected before and after treatment to evaluate the expression levels of *IL4*, *GATA3*, *IL17* and *RORγt*. The gene expression results showed that M2000 has a potent efficacy, so that it could not only significantly decrease *IL17* and *RORγt* levels but also increase *IL4* and *GATA3* levels after 12 weeks of treatment. Moreover, the gene expression results were in accordance with the clinical and preclinical assessments. In conclusion, M2000 as a natural novel agent has therapeutic and immunosuppressive properties on RA patients (identifier: IRCT2014011213739N2).

**Keywords:** M2000, *IL17*, *RORγt*, rheumatoid arthritis, inflammation, immunosuppressive

## Introduction

Alginic acid as a biomaterial has been successfully used in biomedical science, bioengineering and especially pharmaceutical application for over 40 years without any significant side effects.[1] It has been applied in drug delivery systems such as controlled release, disintegrant, film former, thickening and stabilization.[2] Moreover, the positive effect, safety and efficacy of sodium alginate on gastric reflux control and wound care have been completely proved.[3] Accordingly, its comonomer β-D-mannuronic acid (M2000), patented (DEU: 102016113018.4), has been shown to have significant therapeutic and anti-inflammatory properties in several experimental models.[4] Since sodium alginate comes from a natural polysaccharide (brown seaweeds), its comonomers have

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been shown to exhibit sufficient safety in pharmaceutical ingredients.[1] In our previous animal experimental models, M2000 has been shown to have notable anti-inflammatory and immunosuppressive effects on multiple sclerosis, nephrotic syndrome, rheumatoid arthritis (RA) and immune complex glomerulonephritis.[5–9] Prior studies have found some characterized molecular mechanisms of this novel drug, including inhibitory effects on the activity of matrix metalloproteinase-2, inhibition of infiltration of immune cells and reduction of IL-6 and antibody production.[10] In the present clinical study, for the first time, we evaluated the effects of M2000 on RA patients since its administration has been reported to be completely safe in animal models.[11]

RA is the most common autoimmune and systemic inflammatory disease characterized by inflammation and deformity of synovial joints, leading to bone and cartilage erosion. There are some common symptoms in RA such as fatigue, low-grade fever, malaise, morning stiffness, headache, loss of appetite and loss of weight.[12]

Considering the molecular and pathological basis of immune-mediated chronic inflammatory disease, there is an interest in approaching to an effective therapy in RA. T-cell has a great role in RA disease; association of human leukocyte antigen (HLA)-DR known as an MHC class II cell surface receptor assigns a great proof that $CD4^+$ T-cells (Th2) are involved in the development of the disease.[13] Among the other types of T-cells, extensive evidence has shown that the subset of proinflammatory T helper cells such as TH17 play a notable role in the progression of RA.[14] Th2 cytokines, especially IL4, may play an important role in anti-inflammatory effects. GATA3 as its transcription factor could strongly mediate the enhancement of *IL4* promoter and regulate *IL4* gene expression.[15] Hereupon, an appropriate RA treatment should meet the increase in GATA3 and IL4 levels. Another transcription factor RORγt is required for the differentiation of Th17 cells and expression of *IL17*. Afterward, IL17-producing $CD4^+$ Th17 cells, as a new subclass of cytokines with highly proinflammatory properties, could have a potential mechanism and role in the autoimmune diseases such as RA. Therefore, a treatment that is able to attenuate the levels of RORγt and IL17 could be able to control the progression of RA.[16]

Currently, five lines of therapy are used in order to control the progression and symptoms of RA in patients, including disease-modifying anti-rheumatic drugs (DMARDs), biologic response modifiers (a type of DMARD), glucocorticoids, non-steroidal anti-inflammatory drugs (NSAIDs) and analgesics (painkillers). All these medications help patients to attenuate symptoms and inflammation; however, in rare cases, the disease can go into the remission phase. On the other side, overuse of these medications can cause serious adverse effects, including damaging blood cells, liver, lungs, kidney and gastrointestinal tract, increasing the risk of some cancers, osteoporosis and muscle weakness.[17–19] Therefore, not only is an effective treatment an essential target in medicine, but overcoming the side effects is also another substantial challenge in this area. Since RA itself is a painful and frustrating condition, it would indeed be difficult to struggle with the side effects of drugs. In this study, based on our previous approaches, M2000 as a natural medicine and novel designed NSAID with in immunosuppressive properties in T-cell-mediated autoimmune diseases was used for those RA patients who had failed response to the other therapies. Afterward, the effect of M2000 on these RA patients was figured out by the changes in the relative gene expression of *IL4*, *GATA3*, *IL17* and *RORγt* since these genes have a great key role in autoimmune diseases such as RA.

# Materials and methods

## Ethics statement

The study was approved by the ethics committee of Tehran University of Medical Sciences (TUMS) and was conducted under guidelines established by the American College of Rheumatology (ACR) and Helsinki manifest and its later amendments or comparable ethical standards. Written informed consent was obtained from all patients.

## M2000 preparation and intake

M2000 as a small molecule ($C_6H_{10}O_7$) with the molecular weight of 194.139 Da was prepared from sodium alginate (Sigma-Aldrich, St Louis, MO, USA) based on the method of Fattahi et al.[11] Subsequently, the purity of M2000 was validated by characterizing the hydrolytic products using Fourier transform infrared (FTIR) spectroscopy and carbon-13 nuclear magnetic resonance ($^{13}$C-NMR) spectroscopy.

## Clinical characterization of patients and control

In this study, 12 patients with acute RA who had inadequate response to conventional drugs were selected for 12 weeks clinical trial. The mean age of the selected patients (females 10, males two) was 45±14.54 years, and the range of disease duration was 1–15 years. Treatment of these patients

with M2000 based on the ACR criteria for RA was started on 16 May 2014 (identifier: IRCT2014011213739N2). At the baseline, although all patients were treated with DMARDs, steroids and TNF-α antagonists, the disease score (disease activity score in 28 joints [DAS28]) was quite high (Table 1). Prior to the enrollment of this clinical trial, the patients were informed of this study and asked to sign an informed consent. Afterwards, the patients received the follow-up clinic appointment at baseline, 4 weeks and 12 weeks at the Department of Rheumatology, Shariati University Hospital, Tehran, Iran, and the Division of Rheumatology Research, Rheumatism Center. Further follow-up was arranged by telephone for assessing the adverse effects of M2000 every week.

During this clinical trial, patients were allowed to use their routine medications, which included methotrexate (15–20 mg weekly), hydroxychloroquine (400 mg daily) and steroids (5–15 mg daily), and also seven of 12 patients received a subcutaneous injection of etanercept (25 mg twice weekly). However, patients were prohibited from using NSAIDs or other pharmacologic treatment during this 12-week follow-up. Based on the preclinical assessment, a minimum dosage (18 mg/kg/d) of M2000 was provided in a gelatinized capsule (500 mg of M2000) for oral administration. Finally, the M2000 capsule (500 mg) was prescribed twice daily for 12 weeks. Moreover, in this trial, we had 12 participants (nine females and three males) who had only used DMARDs without M2000 as the control (conventional) group, and also 12 healthy subjects (nine females and three males) without any background disease were selected as a normal group for immunological assessment.

## Sample preparation

Blood was obtained from normal donors and patients before and after treatment. Afterward, peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) and stored at −80°C. Total RNA was extracted from $2\times10^6$–$5\times10^6$ cells using GeneAll® Hybrid-RTM kits (Qiagen, Valencia, CA, USA) based on manufacturer's instructions and placed into 50 µL of RNase-free water. The concentration of total RNA was measured by NanoDrop 2000 UV–Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). It was then concentrated or diluted to the concentration <300 ng/µL for cDNA synthesis.

## Quantitative real-time polymerase chain reaction (PCR)

Reverse-transcribed random hexamer primers were used for conventional PCR based on cDNA Synthesis Kit protocol (Takara Co., Ltd., Dalian, China). Quantitative real-time PCR was performed using SYBR® Premix Ex Taq™ II (Takara Co., Ltd.) with specific primer (Sigma-Aldrich; Table 2) based on the provided guideline. The analysis of *IL4*, *GATA3*, *IL17*, *RORγt* and *β-actin* transcripts was carried out in StepOne™ and StepOnePlus™ Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA). The relative quantities of target gene mRNA compared against one internal control, *β-actin* mRNA, were measured by following a $\Delta C_T$ method using an amplification plot (fluorescence signal vs cycle number). The difference ($\Delta C_T$) between the mean values in the replicate samples of target genes and *β-actin* mRNA was calculated. Subsequently, the difference ($\Delta\Delta C_T$) between changes in the expression of target genes and the normal group was calculated and expressed as $2^{-\Delta\Delta C_T}$.

## Statistical analysis

Data were representative of three independent experiments. The data were expressed as mean ± standard deviation, and the analysis was performed by SPSS software (19.0; IBM Corporation, Armonk, NY, USA). Moreover, the parametric data were subjected to analysis of variance (ANOVA) and the Newman–Keuls test to determine significant differences in the gene expression level of before and after treatment. The statistical significance was classified as $^*P<0.05$, $^{**}P<0.01$ or $^{***}P<0.001$. A $P$-value of <0.05 was considered as statistically significant.

**Table 1** Variations in clinical manifestation of 12 RA patients before and after M2000 therapy and control group

| Characteristics | Before (n=12) | After (n=12) | Control (conventional; n=12) | *P*-value |
|---|---|---|---|---|
| DAS28 | 5.8±0.58 | 2.43±0.75 | 5.74±0.18 | <0.003 |
| Morning stiffness (minutes) | 45.38±14.2 | 23.46±12.67 | 40±8.94 | <0.005 |
| RF (IU/mL) | 143.3±1.7 | 53.06±6.3 | 128±46.57 | <0.01 |
| CRP (mg/L) | 17.02±2.4 | 11.97±1.9 | 25.52±7.19 | <0.02 |

**Note:** Values are shown as mean ± SD.
**Abbreviations:** RA, rheumatoid arthritis; DAS28, disease activity score in 28 joints; RF, rheumatoid factor; CRP, C-reactive protein.

**Table 2** *IL4*, *GATA3*, *IL17*, *RORγt* and *β-actin* real-time PCR primers

| Name | Primer sequence | Reference |
|---|---|---|
| *IL17* Fwd | 5′-GAAGGCAGGAATCACAATC-3′ | 33 |
| *IL17* Rev | 5′-GCCTCCCAGATCACAGA-3′ | |
| *RORγt* Fwd | 5′-CTGCTGAGAAGGACAGGGAG-3′ | 34 |
| *RORγt* Rev | 5′-AGTTCTGCTGACGGGTGC-3′ | |
| *IL4* Fwd | 5′-GCCACCATGAGAAGGACACT-3′ | 35 |
| *IL4* Rev | 5′-ACTCTGGTTGGCTTCCTTCA-3′ | |
| *GATA3* Fwd | 5′-GACCCACCACCCCATCA-3′ | 36 |
| *GATA3* Rev | 5′-GGTTCTGTCCGTTCATTTTGT-3′ | |
| *β-actin* Fwd | 5′-GCATGGGTCAGAAGGATTC-3′ | – |
| *β-actin* Rev | 5′-GTCCCAGTTGGTGACGAT-3′ | |

**Abbreviations:** PCR, polymerase chain reaction; Fwd, forward; Rev, reverse.

# Results

## Patient's response

The improvement in the patient status was observed after 2 weeks and also continued during the treatment course. The mean of disease activity (DAS28), morning stiffness, rheumatoid factor (RF) and C-reactive protein (CRP) had met a significant reduction after 12 weeks of treatment (Table 1). In addition, improvement was observed in the other clinical laboratory tests, including anti-CCP, anti-dsDNA and ESR levels, which returned to the normal range.

## Effects of M2000 on *IL4*, *GATA3*, *IL17* and *RORγt* gene expressions

The results represented that after 12 weeks therapy with M2000, *IL17* and *RORγt* gene expressions in patient's, PBMCs were decreased by 22.39- and 2.36-fold, respectively, in comparison to the gene expressions of the patients before therapy. As shown in Figures 1 and 2, after treatment,

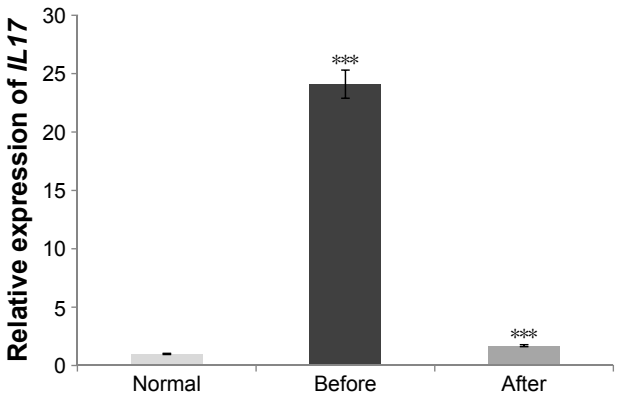


**Figure 1** *IL17* gene expression in normal, before and after treatment groups with M2000.

**Notes:** RNA extraction from PBMCs and quantitative reverse transcription PCR were performed to evaluate the variation of *IL17* gene expression in the above-described conditions. Data are representative of three independent qPCR experiments (level of significance: ***$P<0.001$).

**Abbreviations:** PBMC, peripheral blood mononuclear cells; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction.

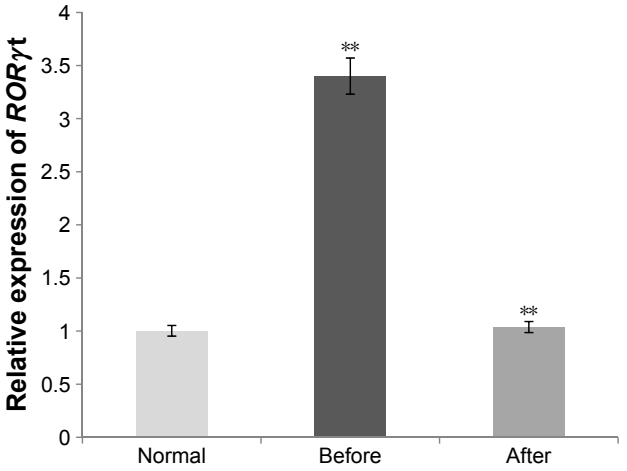


**Figure 2** *RORγt* gene expression in normal, before and after treatment groups with M2000.

**Notes:** RNA extraction from PBMCs and quantitative reverse transcription PCR were performed to evaluate the variation of *RORγt* gene expression in the above-described conditions. Data are representative of three independent qPCR experiments (level of significance: **$P<0.01$).

**Abbreviations:** PBMC, peripheral blood mononuclear cells; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction.

*IL17* and *RORγt* fold changes were 1.7 and 1.03, respectively, which were approximately near to the normal range. Furthermore, the statistical analyses revealed that the differences between the control group and other groups were significant ($P=0.0001$ and $P=0.034$, respectively).

On the other side, the *IL4* and *GATA3* gene expressions in patients' PBMCs were increased by 0.92- and 0.48-fold, respectively, in comparison to the gene expressions of the patients before therapy. Furthermore, in the normal group, the fold change was 1.9 and 1 for *IL4* and *GATA3*, respectively. However, the statistical analyses revealed that the differences between control group and other groups for *IL4* and *GATA3* (Figures 3 and 4) were not significant ($P=0.165$ and $P=0.084$, respectively). The results also demonstrated that although they had a reduction in the expression level, it was not sufficient.

# Discussion

In RA, the immune system is misdirected and attacks the joints. This misdirecting could promote inflammation not only in joints but also in other various organs and tissues of body, occasionally.[20] Generally, the conventional DMARDs and TNF-α blockers were used to reduce both disease activity and progression in patients.[21,22] However, some patients do not respond to these treatments and present a diminished response to them overtime. Furthermore, RA patients are always suffering from the adverse effects of these chemicals and biological drugs.[23] Therefore, M2000 as a novel and

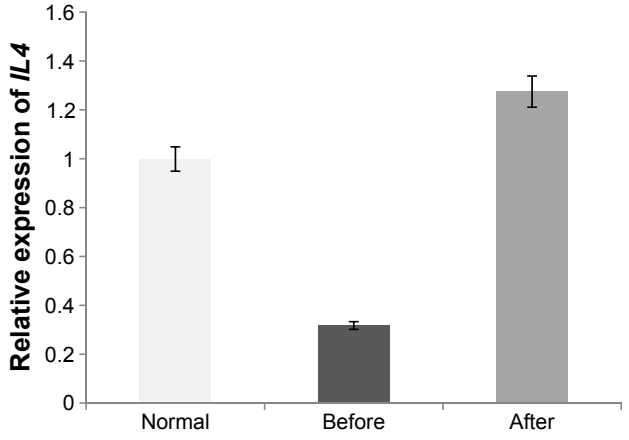


**Figure 3** *IL4* gene expression in normal, before and after treatment groups with M2000.
**Notes:** RNA extraction from PBMCs and quantitative reverse transcription PCR were performed to evaluate the variation of *IL4* gene expression in the above-described conditions. Data are representative of three independent qPCR experiments.
**Abbreviations:** PBMC, peripheral blood mononuclear cells; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction.

natural anti-inflammatory agent was used during 3 months clinical trial and showed a suitable response in the proposed gene expression and clinical and paraclinical results.

In RA, autoimmune response is involved by immune cells, including various white blood cells, such as B- and T-cells, neutrophils, dendritic cells, macrophages and mast cells, which focus on the joint cavity. These cells release a wide range of chemical agents such as inflammatory mediators, cytokines, leukotrienes and prostaglandins.[24–26] These chemicals develop the growth of blood vessels, which supply nutrients for the growth of leukocytes and

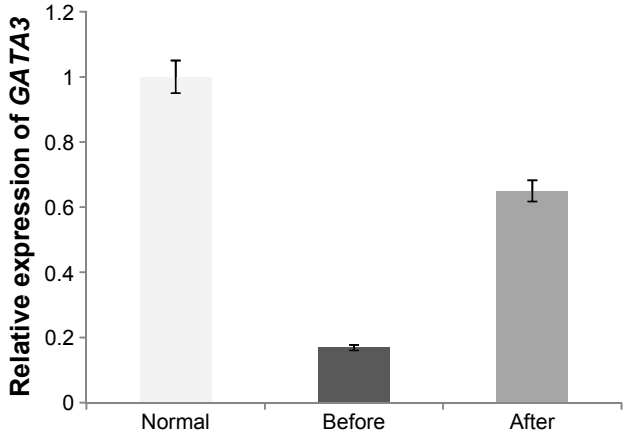


**Figure 4** *GATA3* gene expression in normal, before and after treatment with M2000 groups.
**Notes:** RNA extraction from PBMCs and quantitative reverse transcription PCR were performed to evaluate the variation of *GATA3* gene expression in the above-described conditions. Data are representative of three independent qPCR experiments.
**Abbreviations:** PBMC, peripheral blood mononuclear cells; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction.

fibrous tissues in synovial cartilage.[27] Among these chemical mediators, IL4 and IL17 as T-cell cytokines and their transcription factors GATA3 and RORγt have a critical role in RA.[15,16] Our presented data indicate that *IL4* and its transcription factor (*GATA3*) are expressed at a low level in the peripheral blood cells of RA patients before treatment. IL4 has anti-inflammatory property and disease-restricting role. The potential function of IL4 level in RA patients using antibody-based delivery of IL4 has been reported. On the other hand, it has been illustrated that the lack of IL4 does not appear to affect the activation of autoreactive T-cells and also could display various courses of disease, including no arthritis, transient mild arthritis and severe arthritis.[15] There are two possible explanations for this variability; first, IL4 could decrease the collaboration of T-cells and B-cells, and the other could refer to the other possible cytokines such as IL13 that may lead IL4 function to a level that appears to change the disease condition.[15,28] Therefore, the reason why in this experiment induction of *IL4* and *GATA3* gene expressions was not significant might be due to the above-mentioned reason.

Our results demonstrated that *IL17* and its transcription factor (*RORγt*) showed a significant reduction in PBMCs. Previous evidence has indicated the critical role of IL17 in RA and that suitable therapies are able to reduce its level. Since the increase of IL17 production and upregulation of Th17 cells which characterized by expression of the *RORγt* are the most common features of RA.[26] Regarding the previous studies, IL17-producing $CD4^+$ T (Th17) cells as a unique T-helper, which is known as T-cell-mediated tissue injury, could promote inflammation by proinflammatory cytokines and chemokines in autoimmune disease.[29–31] In the present study, the *IL17* level after treatment with M2000 significantly decreased 22.39-fold the level of these cytokines compared to the before treatment group. The results of this trial are in agreement with the other studies that reported the potential role of IL17 in mediating joint damage and the ability of IL17 to induce collagen release from cartilage.[31,32] In this study, there were significant correlations between the gene expression results and clinical and paraclinical assessments. The levels of these cytokines were in accordance with the disease activity (DAS28), tender joint and swelling, all of which showed a reduction in mean after treatment. Moreover, the range of ESR and CRP, which was quite high before M2000, faced a significant reduction and back to the normal range.

This clinical trial revealed potent anti-inflammatory and immunosuppressive properties of M2000 in 12 RA patients. During this trial, due to the good response of patients to this natural agent, the rheumatologist started to reduce the

corticosteroid dosage, and at the end of 3 months, the intake dosage of corticosteroid was decreased to the half. In addition, etanercept injection was eliminated in patients.

## Conclusion

Finally, based on the clinical assessment and genes expression results, it can be concluded that oral administration of M2000 as a novel NSAID with immunosuppressive property and a natural agent could help in the process of RA treatment. Moreover, this finding brings a perspective view for further trials with a higher number of patients and also other areas in autoimmune diseases.

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## Disclosure

The authors report no conflicts of interest in this work.

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