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## Hematological Improvement of Patients with Active Rheumatoid Arthritis by $\beta$ -D-Mannuronic Acid (M2000) as a Novel NSAID with Immunosuppressive Property

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### ABSTRACT

The aim of this study was to investigate the effect of  $\beta$ -D-mannuronic acid (M2000) on hematological parameters in patients with active rheumatoid arthritis.

This study was conducted on 25 patients with active rheumatoid arthritis (RA) (identifier: IRCT2014011213739N2). M2000 was administered orally for anemic and non-anemic RA patients at a dose of 500 mg twice daily for 12 weeks. The patients were permitted to continue the conventional treatments excluding NSAIDs. Blood samples were collected at baseline, 4 and 12 weeks after drug administration and were tested for hematological parameters. Moreover, serum levels of TNF- $\alpha$  and IL-6 were analysed before and after M2000 therapy compared to healthy controls using enzyme linked immunosorbent assay method.

We found a significant increase in the count of red blood cells and also hemoglobin (Hb) concentration (0.9 g/dL) in anemic patients after 12 weeks of M2000 therapy ( $p < 0.02$  and  $p < 0.01$ , respectively). Furthermore, our results showed an improvement in Hb level (0.45 g/dL) even in non-anemic patients who were treated by M2000 ( $p < 0.04$ ). The leukocytosis in RA patients, significantly decreased in both anemic and non-anemic patients after 12 weeks of M2000 therapy ( $p < 0.02$  and  $p < 0.03$ , respectively). The percent of neutrophils significantly increased in anemic patients ( $p < 0.01$ ) while in non-anemic patients it significantly decreased after 12 weeks of M2000 therapy ( $p < 0.01$ ). The serum levels of IL-6 and TNF- $\alpha$  significantly decreased after 12 weeks of M2000 therapy ( $p < 0.01$  and  $p < 0.04$ , respectively).

M2000 improves hematological parameters in RA patients by its potent inhibitory effect on serum levels of TNF- $\alpha$  and IL-6.

**Keywords:** Anemia; Inflammation; Interleukin; Mannuronic acid; M2000; Rheumatoid arthritis; Therapy

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## INTRODUCTION

Anemia with the prevalence of 30 to 70 percent is one of the most infirm extra-articular complications in patients with rheumatoid arthritis (RA) which is accompanied by disease activity, radiological progression of disease and increased mortality.<sup>1-3</sup> Hematological abnormalities in RA are multifactorial and characterized by anemia, neutropenia, thrombocytosis, eosinophilia, and hematological malignancies.<sup>4</sup> Anemia of chronic disease (ACD) and iron-deficiency anemia (IDA) are mainly the cause of anemia in RA.<sup>5</sup> The principal mechanisms of ACD in RA is still unknown but the inflammatory cytokines such as IL-6 and tumor necrosis factor (TNF- $\alpha$ ) with effect on iron regulatory hormone named hepcidin and are associated with the impairment of erythropoiesis as well as blunted erythropoietin response.<sup>6-9</sup> The therapeutic effect of iron therapy in patients with anemia of chronic disease is not unequivocal.<sup>5</sup> Hepcidin, by retention of iron into the reticuloendothelial and decrease in iron absorption from duodenum is involved in the pathophysiology of ACD.<sup>10,11</sup>

Biological therapies such as tocilizumab (TCZ) and TNF- $\alpha$  blockers have emerged as a milestone in the treatment of RA, especially in patients with resistant to conventional treatment. These treatments cause an increase in the mean haemoglobin (Hb) concentration after treatment.<sup>12-14</sup> However; the use of anti-TNF- $\alpha$  therapy is accompanied with serious adverse effects including increased risk of lymphoma, hematologic disorders and mortality. Therefore, it has been established a major safety concern in patients undergoing treatment with biological therapy.<sup>15</sup> Methotrexate (MTX) as a folate antagonism induces hematological toxicities including bone marrow suppression and pancytopenia which in some cases can be fatal.<sup>16</sup> Moreover, the protective effect of folate supplementation for the prevention of MTX-induced hematological toxicity is controversial.<sup>17</sup>

The recombinant erythropoiesis-stimulating agents (ESA), such as human erythropoietin (alpha and beta) and darbepoetin alpha have been recommended in treatment of chronic anemia in RA whereas, blunted erythropoietin response even in the presence of normal erythropoietin and the persistent chronic inflammation have suppressed the mechanisms of erythropoiesis.<sup>18,19</sup>

Although, the considerable developments have been

made in the understanding of molecular mechanisms of anemia, however unsatisfactory progression has been attained in the treatment of anemia.<sup>3</sup> Therefore; the control of inflammation in these patients could be a pivotal treatment option in ACD and IDA.

The low molecular weight,  $\beta$ -D-mannuronic acid (M2000) (DE-102016113018.4) is one of the Alginate derivatives, which has shown the marked therapeutic effects on several experimental models including experimental autoimmune encephalomyelitis (EAE), nephrotic syndrome, immune complex glomerulonephritis and adjuvant induced arthritis (AIA).<sup>20-24</sup>

The administration of  $\beta$ -D-mannuronic acid was associated with a significant reduction in the paw edema and matrix metalloproteinase (MMPs) activity and prevented from the development of joint destruction in adjuvant induced arthritis model.<sup>21</sup> This drug also significantly reduced the production of serum cytokine IL-6, in the experimental model of nephritis.<sup>24</sup>

In the present study that was carried out for the first time, we determined the effect of this new anti-inflammatory drug, M2000 on the hematological parameters of RA patients with or without anemia in active stage of the disease who had inadequate response to treatment. Therefore, in this study we analyzed the hematological parameters of the patients at baseline, 4 weeks and 12 weeks after treatment. Furthermore, we also evaluated the changes of inflammatory cytokines including TNF- $\alpha$  and IL-6 in all of RA patients before and after of M2000 therapy.

## MATERIALS AND METHODS

### Extraction of $\beta$ -D-Mannuronic Acid

The low molecular weight,  $\beta$ -D-mannuronic acid (M2000), patented (DE-102016113018.4) was prepared and extracted from Alginate sodium salt (Sigma-Aldrich, St. Louis, MO, USA) based on the Fattahi and co-workers protocol<sup>25</sup>. This method of extraction was confirmed and controlled using nuclear magnetic resonance (NMR-<sup>13</sup>C) spectroscopy and Fourier Transform Infrared (FT-IR) spectroscopy.

### Patients and Methods

This trial study was conducted on 25 patients with RA for 12 weeks after fulfilling the American College of Rheumatology (ACR) criteria for RA.<sup>26</sup> The study was performed in accordance with the declaration of

Helsinki following to get an official approval by the ethics committee of Tehran University of Medical Sciences (No. 92/D/130/2392) and it was registered on May 16, 2014 (identifier: IRCT2014011213739N2).

At baseline, the patients were classified in two groups (patients with anemia and patients without anemia). 13 of the patients had the manifestation of anemia and 12 patients, without the characteristics of anemia. In the initiation of study the clinical data included age, gender, disease duration, anti-CCP positivity, RA positivity were recorded.

At baseline all of patients had high disease activity and the anemic patients despite of concomitant treatment by iron drugs and folic acid had the features of anemia. The concomitant treatment with a stable dose of other medications including methotrexate, glucocorticoids, hydroxychloroquine, etanercept, iron drugs and folic acid excluding non-steroidal anti-inflammatory drugs (NSAIDs) were allowed during 12 weeks of follow up.

According to preclinical assessment the approved selected dosage of  $\beta$ -D-mannuronic acid (M2000) was 25mg/kg/d but in this study we used a minimum dose (18mg/kg/d) of M2000.<sup>26</sup> M2000 was prepared as gelatinized capsules and all of patients were treated orally at a dose of 500 mg twice daily for 12 weeks after obtaining of written informed consent. The patients were visited at baseline, 4 weeks, and 12 weeks at the Rheumatology Research Center, Shariati Hospital and Iran Rheumatism Center during 12 weeks of follow up.

The characteristics of disease such as Disease Activity Score (DAS28) based on the number of tender and swollen joints in 28 joints, the erythrocyte sedimentation rate (ESR), and the global health assessment were recorded at base line, 4 weeks and after 12 weeks of follow up.<sup>27</sup>

The assessment for anemia included the determination of red blood cell count (RBC), mean Hb concentration, total white blood cell and platelet count; red blood cell parameters such as mean corpuscular volume (MCV), mean corpuscular Hb concentration (MCHC) and mean corpuscular Hb (MCH). Anemia was defined as Hb level <12.0 in women and <13.0 in men. In addition, the percent of neutrophils, lymphocytes, monocytes, eosinophils and basophils were determined with an electronic cell counter.

The patients who had malignancies, renal failure, gastrointestinal bleeding, hemolytic conditions or

receiving erythropoietin therapy were excluded from the study. After screening of personal and family history for RA, fifteen healthy persons who were matched in sex, age and body mass index (BMI) were selected from the staffs of Tehran University of Medical Sciences (TUMS).

### Sample Collection and Cytokine Measurements

The blood samples were obtained from antecubital vein of the patients according to Ethical Committee permissions at Iran Rheumatism Center by a vacutainer, (Becton Dickinson, Plymouth, UK) and were put at room temperature for clotting. The serum samples of the patients and the healthy volunteers were isolated by centrifugation (15 min, 1500 g). Serum samples were stored at  $-70^{\circ}\text{C}$  until TNF- $\alpha$  and IL-6 assessment. The serum level of TNF- $\alpha$  and IL-6 were measured by a commercially enzyme-linked immunosorbent assay (ELISA) kit (eBioscience, San Diego, USA). The minimum detectable level for IL-6 and TNF- $\alpha$  ELISA assay were  $0.04\text{ pg ml}^{-1}$  and  $0.09\text{ pg ml}^{-1}$ , and also had performance sensitivity of less than  $0.16\text{ pg ml}^{-1}$  and  $0.5\text{ pg ml}^{-1}$ , respectively. The assessments were done according to the instructions of manufacturer and all assays were carried out in duplicate for each sample and optical density of microplates were read by ELISA reader at wavelength of 450 nm. The laboratory parameters including erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) were measured by the westgren and immunoturbidimetry assay (Audit Diagnostics, Carrigtwohill, Ireland), respectively. Furthermore, after taking blood sample from each person, blood specimens were collected in the tubes with concentration of 1.5 mg/ml of ethylene di-amine tetra acetic acid (EDTA) and Hb concentration in all samples was determined according to the cyanomethaemoglobin method using an electronic cell counter (Sysmex coulter counter, USA).<sup>28</sup>

### Statistical Analysis

Mann-Whitney U-test was used for the assessment of continuous variables in two groups and frequencies were analyzed by Chi-square test. Data were shown as the median and range. The variations from baseline to after of treatment in each group were assessed by Wilcoxon's signed rank test. Statistical analysis was carried out by SPSS software, version 11.0.1 J (SPSS, Inc., Chicago, IL, USA)  $p < 0.05$  was considered

statistically significant.

### RESULTS

The baseline demographic and clinical variables of the patients groups are summarized in Table 1. 13 out of 25 patients had the manifestation of anemia. The median age of RA patients for anemic and non anemic patients were 47(26 to 69) and 45.5 (26 to 69) years in anemic and non anemic patients, respectively. The median disease duration of patients in both groups were similar 4 years in anemic (1 to 20) versus 4.5 (1 to 18) years in non anemic patients, respectively. Eleven anemic RA patients had features of anti-CCP positive while ten patients were positive for anti-CCP in non anemic group. Furthermore, ten patients in anemic group were positive for RF whereas, eight patients in non anemic group were positive for it.

All of patients in both groups were treated by disease-modifying anti-rheumatic drugs (DMARDs), prednisolone, folic acid and also five patients in anemic and two patients in non anemic group were under treatment of anti-TNF $\alpha$  therapy (etanercept). The NSAIDs were being used by all of patients but after the beginning of the study were removed from the

treatment regimen of patients. Moreover, seven patients in anemic group were used iron drugs. RA patients with anemia, significantly had severe disease activity score (DAS28), 6.21 (5.26 to 7.19) compared with non anemic patients 5.25 (5.18 to 6.88) ( $p<0.02$ ).

### The Effect of M2000 Therapy on RBC and Related Indices

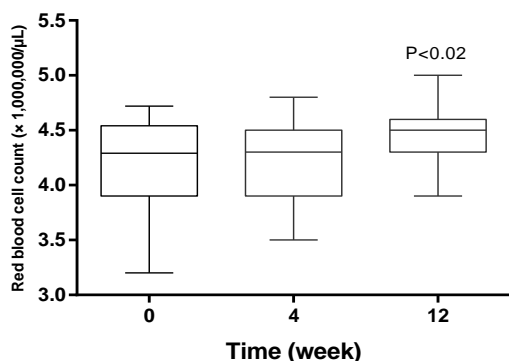
The median level of Hb concentration in patients with anemia was 11.4 g/dL whereas the level of red blood cell (RBC) counts and the values of HCT, MCV, MCH and MCHC were lower in anemic patients than non-anemic patients. The platelet counts were higher in anemic patients compared to non-anemic patients (Table 2). The level of red blood cell counts increased progressively in both of two groups after 4 weeks of treatment and remained significant in anemic group from 4.29 to 4.5 ( $\times 10^6/\mu\text{L}$ ) in anemic patients after 12 weeks of M2000 therapy ( $p<0.02$ ) (Figure 1).

We observed a significant improvement in the level of Hb concentration (0.9 g/dL) in anemic patients (11.4 g/dL at onset vs. 12.3 g/dL) after 12 weeks of M2000 therapy ( $p<0.01$ ) (Figure 2). Furthermore, we also found significant increase in the level of Hb concentration (0.45 g/dL) even in non-anemic patients

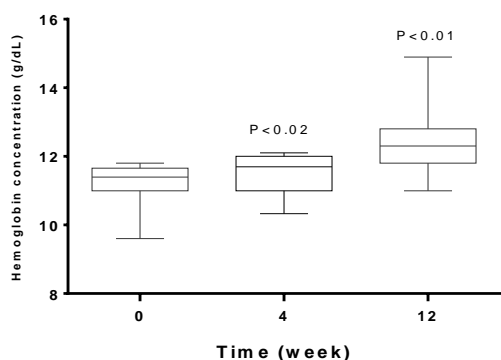
**Table 1. Baseline demographic characteristics of 25 rheumatoid arthritis patients with anemia and without anemia**

Characteristics	Anemic patients n=13	Non-anemic patients n=12	p value
Age (years)	47 (26-69)	45.5 (28-70)	NS
Women (%)	93.3% (12)	81.8% (10)	NS
Duration of disease (years)	4 (1-20)	4.5 (1-18)	NS
Anti-CCP positivity, % (n)	84.6 (11)	83.3% (10)	NS
RF positivity, % (n)	76.9 (10)	66.6 (8)	NS
ESR (mm/h)	37 (12 to 105)	25 (10 to 91)	NS
CRP (mg/dl)	15 (3.8 to 92)	8.1 (2 to 30)	$p<0.04$
Concomitant treatments:			
DMARDs, % (n)	100% (13)	100% (12)	-
Previous NSAIDs usage, % (n)	100% (13)	100% (12)	-
Prednisolone, % (n)	100% (13)	100% (12)	-
Etanercept, % (n)	33.3% (5)	20% (2)	-
Folic acid, % (n)	100% (13)	100% (12)	-
Iron drugs, % (n)	53.8 (7)	-	-
DAS28	6.21 (5.26 to 7.19)	5.25 (5.18 to 6.88)	$p<0.02$

Continuous variables were analysed by Mann-Whitney U-test and were presented as median (min-max). Chi square test was used to comparison of categorical variables and were presented as % (number) unless otherwise mentioned. Anti-CCP, anti-cyclic citrullinated peptide antibodies; RF, rheumatoid factor; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; DMARD, disease modifying anti-rheumatic drug. NSAIDs, non-steroidal anti-inflammatory drugs; DAS, disease activity score; NS, not significant



**Figure 1.** Changes in red blood cells count of anemic patients with rheumatoid arthritis during 12 weeks of M2000 therapy. Data presented as median and range. The lines in the boxes indicate median and the edges of the boxes show min and max points



**Figure 2.** Changes in the level of haemoglobin (Hb) concentration in anemic patients with rheumatoid arthritis during 12 weeks of M2000 therapy. The Hb level (g/dL) was presented as median and range. The lines in the boxes indicate median and the edges of the boxes show min and max points.

(from 12.75 g/dL at onset vs. 13.2 g/dL) who were treated by M2000 ( $p < 0.04$ ) (Table 2). Five of thirteen anemic patients recovered from anemia and returned to reference range and other patients, showed an increase in Hb concentration. We also found a significant improvement in the percent of HCT in anemic (from 34.2% at onset vs. 37.8%) and non-anemic patients ( $p < 0.01$  and  $p < 0.03$ , respectively) (Table 2). Furthermore, M2000 therapy showed an improvement in the values of MCV, and MCHC along with the increase in the amount of Hb concentration in anemic

patients after 12 weeks of M2000 therapy ( $p < 0.03$ , and  $p < 0.01$ , respectively). Meanwhile, there was also an improvement in the values of MCV, MCH and MCHC in non-anemic patients (Table 2).

At the beginning of study, 4 (30.7%) patients with anemia had the manifestation of thrombocytosis. Following treatment with M2000 the platelet counts in these patients were reduced ( $p < 0.04$ ) and returned to normal range (Table 2).

### The Effect of M2000 on WBC and Related Indices

At base line, both of two groups had elevated level of white blood cell (WBC) counts. The level of WBCs, significantly decreased throughout in both anemic and non-anemic patients after 12 weeks of M2000 therapy ( $p < 0.02$  and  $p < 0.03$ , respectively) whereas the rate of reduction was greater in anemic patients from 11.9 at onset vs. 8 ( $\times 10^3/\mu\text{L}$ ) compared to non-anemic patients (Figure 3).

The percent of neutrophils significantly increased in anemic patient ( $p < 0.01$ ) while in non-anemic patients its percent significantly decreased after 12 weeks of M2000 therapy ( $p < 0.01$ ). Moreover, the percent of lymphocytes significantly decreased in anemic patients ( $p < 0.03$ ) compared to non-anemic patients after 12 weeks of M2000 therapy (Table 2).

The count of monocytes and eosinophils in anemic group decreased significantly after 12 weeks of M2000 therapy ( $p < 0.03$  and  $p < 0.01$ , respectively). Furthermore, we did not observed any significant change in the count of basophiles in both groups after 12 weeks of M2000 therapy (Table 2).

### The Change in Serum Levels of IL-6 and TNF- $\alpha$ During M2000 Therapy

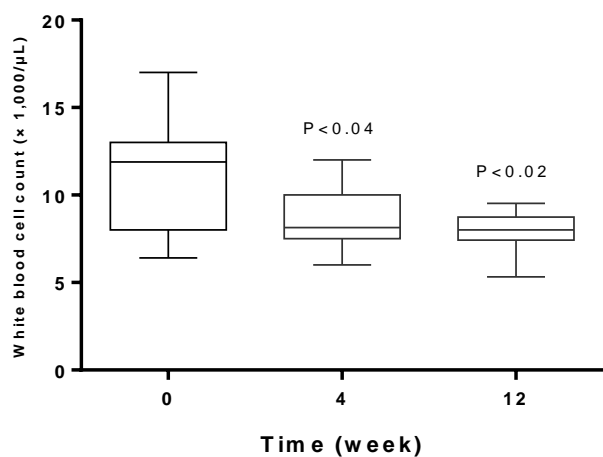
The serum levels IL-6 and TNF- $\alpha$  were measurable in all of control subjects and patients. At baseline, in all of patients the median serum levels of IL-6 and TNF- $\alpha$  were 20 (9.11 to 198.8)  $\text{pg mL}^{-1}$  and 68.69 (6.2 to 418.9)  $\text{pg mL}^{-1}$ , respectively and the amount of serum level in patients significantly were higher in compared with control subjects (median 6.52 and 7.04  $\text{pg mL}^{-1}$ , respectively) with ( $p < 0.001$  and  $p < 0.001$ ) (Figure 4).

In total, we observed that the median serum levels of both IL-6 and TNF- $\alpha$  significantly decreased from 20 to 12.16  $\text{pg mL}^{-1}$  and 68.69 to 45.7  $\text{pg mL}^{-1}$ , respectively after 12 weeks of M2000 therapy ( $p < 0.01$  and  $p < 0.04$ ) (Table 3).

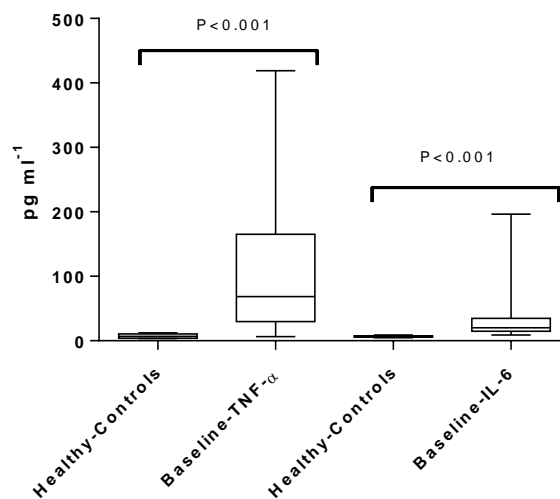
**Table 2. Effect of M2000 therapy on hematological parameters in rheumatoid arthritis patients with anemia and without anemia**

Parameters	Anemic patients n = 13		p value	Non-anemic patients n = 12		p value
	Weeks 0	Weeks 12		Weeks 0	Weeks 12	
RBCs (x 10 <sup>6</sup> /μL)	4.29 (3.2 to 4.72)	4.5 (3.9 to 5)	<0.02	5.12 (4.2 to 6.2)	5.15 (4.26 to 6.3)	NS
Hb (g/dL)	11.4 (9.6 to 11.8)	12.3 (11 to 14.9)	<0.01	12.75 (12 to 17.6)	13.2 (12.8 to 17.6)	<0.04
HCT %	34.2 (28.9 to 35.5)	37.8 (33.1 to 44.7)	<0.01	38.35 (36.1 to 43.5)	39.7 (38.4 to 52.8)	<0.03
MCV (fl)	82 (67.3 to 86.8)	84 (67 to 91)	<0.03	89.3 (85 to 94.8)	90.9 (83.9 to 104)	<0.03
MCH (pg)	24 (20.1 to 29)	27 (23 to 30)	NS	28.3 (27.3 to 31.5)	32 (28 to 34)	<0.01
MCHC %	29 (23 to 32)	32.1 (29 to 34.8)	<0.01	32.1 (30 to 35.28)	33.2 (32 to 36)	NS
Platelet (10 <sup>3</sup> /mL)	414 (206 to 668)	315 (210 to 375)	<0.04	268 (176 to 333)	277 (215 to 380)	<0.01
WBCs (x 10 <sup>3</sup> /μL)	11.9 (6.4 to 17)	8 (5.32 to 9.52)	<0.02	7.89 (5.17 to 12.4)	7 (4.4 to 9.62)	<0.03
Neutrophils %	54 (47.6 to 60.4)	58.55 (56 to 76.5)	<0.01	70 (54.3 to 83)	59 (48.4 to 73)	<0.01
Lymphocytes %	38.25 (23 to 51)	30 (23.4 to 35.3)	<0.03	30 (14 to 43)	29.8 (20 to 38)	NS
Monocytes %	8.8 (5 to 12.4)	6.4 (2 to 10.9)	<0.03	6.2 (3 to 10.7)	4.8 (2 to 7.5)	NS
Esinophils %	3.3 (2.5 to 7.5)	1.6 (0.3 to 3.3)	<0.01	1.9 (0.4 to 2.8)	1.15 (0.2 to 2.7)	NS
Basophils %	0.3 (0.2 to 0.7)	0.3 (0.2 to 0.6)	NS	0.35 (0.1 to 0.6)	0.4 (0.1 to 0.6)	NS

Values are presented as median and range for both groups. Wilcoxon's signed rank test was used. RBC, red blood cell count; Hb, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; WBC, white blood cells. NS, not significant



**Figure 3. Changes in white blood cells count of anemic patients with rheumatoid arthritis during 12 weeks of M2000 therapy. Data presented as median and range. The lines in the boxes indicate median and the edges of the boxes show min and max points.**



**Figure 4. Comparison of IL-6, and TNF-α levels in 25 rheumatoid arthritis patients and 15 healthy controls. Data are presented as median and range. The lines in the boxes indicate median and the edges of the boxes show min and max points**

**Table 3. Variations in the serum levels of IL-6 and TNF- $\alpha$  in rheumatoid arthritis patients before and after 12 weeks of M2000 therapy**

Parameters	Before M2000 therapy (n = 25)	After 12 weeks of M2000 therapy (n = 25)	p value
IL-6 (pg/mL)	20 (9.11 to 198.8)	12.16 (4.12 to 172)	<0.01
TNF- $\alpha$ (pg/mL)	68.6 (6.2 to 418.9)	45.7 (2.1 to 365.8)	<0.04

IL, interleukin; TNF, tumour necrosis factor.

Wilcoxon's signed rank test was used. Data are presented as median and range.

## DISCUSSION

Anemia of chronic disease usually is present in 75 percent of RA patients and positively is related with articular inflammation and increased level of inflammatory cytokines.<sup>1-4</sup> The inflammatory cytokines such as IL-6 play a pivotal role in the production of increased amount of hepcidin from the hepatocytes.<sup>7</sup>

The increased internalization of ferroportin molecules by hepcidin, prevents iron absorption in the intestine, iron releasing from iron reservoirs like macrophages and also with inhibitory effect on the bone marrow blunting the erythropoiesis by decreasing respond to erythropoietin.<sup>7,9</sup> Furthermore, NSAIDs are responsible of the most important drug-induced toxicity with the incidence of at least 16,500 NSAID-related deaths among patients with RA in each year. Moreover, between 10 to 40 percent of drug users have the progressive gastric ulcers and 5-15% duodenal ulcers which are associated with the development of IDA in RA.<sup>29, 30</sup>

Regarding, the persistent chronic inflammation and drug-induced toxicity in RA patients which suggest an advancement in the developing of safe and more specific anti-inflammatory drugs with erythropoiesis-stimulating property, we designed this study.

The  $\beta$ -D-mannuronic acid (M2000) as a novel anti-inflammatory drug with unique properties such as great tolerability, safety and biocompatibility is associated with marked therapeutic effects on several animal models including experimental autoimmune encephalomyelitis (EAE), nephrotic syndrome, acute glomerulonephritis and adjuvant induced arthritis (AIA).<sup>20-24</sup>

The study of preclinical assessment showed that the treatment with  $\beta$ -D-mannuronic acid at the levels up to

1250 mg/kg/d is safe without any complication and toxicity.<sup>25</sup> Based on these results we conducted our current clinical trial on anemic and non-anemic patients with RA.

With respect to the fact that, white and red blood cells are produced from the same stem cells in the bone marrow, the up-regulation of white blood cells in the bone marrow is under the influence of inflammatory cytokines such as IL-1 $\beta$  which is lost associated with the down regulation in producing of red blood cells and the reduction of erythropoiesis even in the presence of normal amounts of erythropoietin.<sup>31,32</sup> Moreover, Dainiak et al; have reported that the presence of serum inhibitor of erythroid colony growth factor in cultured bone marrow cells in RA patients with anemia is related to disease activity and suppression of erythroid precursors stem cells.<sup>33</sup>

During 12 weeks trial study which was carried out for the first time on RA patients with inadequate response to anti-anemic treatments (folic acid and iron drugs), we showed the anti-anemic properties of this novel anti-inflammatory drug in patients with anemia. Our trial revealed a potent significant increase in RBCs counts in blood of anemic patients which was associated with significant decrease in the count of abnormal increased white blood cells.

Our study also revealed a significant improvement in the percent of HCT and the concentration of Hb (0.9 g/dL) which was comparable with the results of a similar study that was carried out by tocilizumab therapy (1.1 g/dL)<sup>34</sup> and also it was associated with a significant improvement in the values of HCT, MCV, and MCHC after 12 weeks of M2000 therapy. Furthermore, we observed an improvement in Hb level and other related indices in non-anemic patients after M2000 therapy.

Our findings provide evidence that M2000 therapy in addition to having anti-anemic effects in anemic patients; could also have a preventative effect on non-anemic patients.

The administration of DMARDs, NSAIDs and other medication in RA patients are associated with drug-induced neutropenia and increased rate to infection especially, in patients with characteristics of diminished neutrophil counts which in mild status is recommended stopping drug, and the use of prophylactic medication and in severe status treatment with G-CSF or GM-CSF.<sup>4</sup>

Our trial showed a significant increase in the neutrophils counts in anemic patients after M2000 therapy. On the other hand, our results also showed that M2000 is able to significantly decrease the elevated neutrophils level in non-anemic patients. Therefore, M2000 along with its anti-anemic effects could modify and adjust the production of neutrophils in the bone marrow. Meanwhile thrombocytosis is usual features of RA with unknown etiology. There is a positive relation between the count of platelet and disease activity along with pulmonary disorders, peripheral neuropathy, and vasculitis [35]. In present study we showed for the first time a significant reduction in the platelet counts of anemic patients after 12 weeks M2000 therapy.

IL-6 has been considered as the most common cytokine in patients with RA with an elevated level in serum and synovial fluid (SF). In these patients, the increased level of IL-6 positively is correlated with disease activity, the pathogenesis of ACD and fatigue in patients with RA.<sup>36</sup>

A previous study has shown that the mice with gene knockouts in IL-6 or hepcidin do not progress anemia<sup>35</sup> and it has been reported that the biological therapies, especially tocilizumab (anti-IL-6 receptor antibody) and anti-TNF $\alpha$  therapy cause an increase in the concentration of Hb level after therapy.<sup>13,14</sup>

It has been reported that M2000 therapy, could significantly reduce the production of serum level of cytokine IL-6 in the experimental model of nephritis.<sup>22,24</sup> In this trial, we observed a significant decrease in the serum levels of IL-6 and TNF- $\alpha$  in RA patients after 12 weeks of M2000 therapy. Therefore, M2000 with inhibitory effect on the production of TNF- $\alpha$  and specially IL-6 is able to reduce hepcidin and finally increase erythropoiesis

In present study that was planned for evaluating the therapeutic effect of this novel drug in RA, the levels of

Hb concentration and RBCs were increased significantly and also observed an improvement in other blood parameters such as HCT, MCV, and MCHC in anemic patients with RA who had failed their treatments for anemia. We also found a significant improvement in hematological parameters of non-anemic patients who were treated simultaneously by M2000. On the other hand, the rate of WBCs reduced in both groups after M2000 therapy. This study revealed that M2000 in addition to having an anti-anemic effect by inhibiting of IL-6 and TNF- $\alpha$  is able to play an adjustment role on the production of cells in the bone marrow. M2000 showed a great tolerability and biocompatibility without adverse effects during M2000 therapy. However, this study was carried out on a limited group of patients with a short time of follow up. Therefore, further time of follow-up with higher number of patients is required for better evaluation of mechanisms and the M2000 efficiency.

Our trial results revealed that M2000 ( $\beta$ -D-mannuronic acid), as a novel anti-inflammatory drug with great tolerability, biocompatibility and without adverse effect has an anti-anemic effect on hematological abnormalities in anemic patients with RA who had failed treatment for anemia. Furthermore, M2000 showed a potent inhibitory effect on serum levels of IL-6 and TNF- $\alpha$  which is associated with an improvement in hematological parameters in anemic and non-anemic patients with RA.

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