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The Anti-tumoral Effect of β -D-Mannuronic Acid (M2000) as a Novel NSAID on Treg Cells Frequency and MMP-2, MMP-9, CCL22 and TGF β 1 Gene Expression in Pre-surgical Breast Cancer Patients

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ABSTRACT

With respect to the role of chronic inflammation in the induction and progression of breast cancer (BC). The relationship between tumor and tumor microenvironment may be a hopeful strategy for BC therapy. According to the effect of β -D-Mannuronic acid (M2000) as a novel non-steroidal anti-inflammatory drug (NSAID) on BC murine model and 4T1 cell line, we started to study that was a phase II, randomized, controlled clinical trial.

24 women with BC were included in this study and were followed by fixed oral doses of M2000, 500 mg two times a day (6-8 weeks). Blood samples were collected at baseline and weeks 6-8. To compare the patterns of matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP-9), C-C motif chemokine ligand 22 (CCL22) and The transforming growth factor-beta 1 (TGF β 1) gene expression and T regulatory cells (Tregs) frequency of healthy women normal controls with BC patients, a set of 10 blood samples of women healthy volunteers was collected. The gene expression was evaluated by quantitative Real-time PCR (qRT-PCR) and the frequency of Tregs was assessed by flow cytometry.

Our results showed, reduction in MMP-2 ($p=0.08$), MMP-9 ($p=0.03$), CCL22 ($p=0.003$) and TGF β 1 ($p=0.1$) gene expression and Tregs frequency ($p=0.01$) which play a main role in the development of chronic inflammation, angiogenesis, tumorigenesis and metastasis.

Our findings demonstrated that M2000 therapy as a novel designed NSAID had valuable therapeutic effects on BC. No adverse effects were observed following the use of M2000 after 6-8 weeks.

Keywords: Breast Cancer; β -D-Mannuronic acid; Chemopreventive; M2000; Non-steroidal anti-inflammatory drug

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INTRODUCTION

1 in 8 women in the United States will develop breast cancer (BC) disease in their life.¹ Based on GLOBOCAN report on 1.38 million new cases were diagnosed in 2008 and it was 1.67 million in 2012, it means 23% of all female cancers contain of BC. The occurrence of BC has increased by 3.1% yearly in the world, between years 1980 to 2010.^{2,3} Surgery, chemotherapy and hormone therapy are presently the main therapeutic options for BC therapy.⁴ However, these approaches are not absolutely effective against metastatic BC, since they show high toxicities, unpleasant side effects, and tumor relapse.⁵ Tumor microenvironment plays a key role in cancer expansion and metastasis. About 15% of human cancers occur from chronic inflammatory diseases.⁶

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases. MMPs not only play the main role in the physiological process but also account for the cancer invasion, metastasis, angiogenesis, and tumorigenesis.^{7,8} It has been reported that BC patients with high MMP-2 and MMP-9 expression showed poor prognosis. The early and exact recognition of the expression level of MMP-9 may provide an effective factor for the prognostic BC therapy.^{9,10}

The transforming growth factor-beta 1 (TGF- β 1) belongs to a superfamily of cytokines. Many studies have reported the overexpression of TGF- β 1 in various types of human cancer, which associates with tumor progression, metastasis, angiogenesis and poor prognostic outcome.^{11,12}

C-C motif chemokine ligand 22 (CCL22), known as macrophage-derived chemokine (MDC), was originally found to be secreted by macrophages and dendritic cells (DC). CCL22 is one of the ligands for chemokine receptor CCR4 that preferentially expresses on T regulatory cells (Tregs) and is required for intra-tumoral Tregs migration.¹³ Some studies have shown that CCL22 secreted by solid tumor cells is responsible for the accumulation of Foxp3⁺ Tregs in BC. This cascade might be led to the suppression of the local immune response and tumor growth. The levels of intra-tumoral Tregs infiltration were related to the levels of TGF- β 1 and CCL22 expression in BC.^{14,15}

Tregs are assumed to play an important role in tumor immunity and investigation on the patterns of its infiltration and its clinical effect on patients has

become an emerging interest. It showed that higher tumor infiltrated lymphocytes (TILs) level was statistically associated with a poor overall survival rate in BC patients.^{16,17}

The patented (DE-102016113018.4) small molecule, β -D-Mannuronic acid (M2000), with molecular formula (C₆H₁₀O₇) was synthesized from alginic acid sodium salt (Sigma-Aldrich, St. Louis, MO, USA) based on the modified procedure of the acid hydrolysis method by Mirshafiey and co-worker in department of immunology, school of public health, Tehran University of Medical Science. The purity of M2000 was confirmed by characterizing the hydrolytic products using Fourier transform infrared (FTIR) spectroscopy and carbon-13 nuclear magnetic resonance (¹³CNMR) spectroscopy for approving its molecular weight (194.139 g/mol). M2000 is an anti-inflammatory pharmaceutical agent with high biocompatibility and no toxicity on functions of the kidney and gastrointestinal (GI) tract.¹⁸ The therapeutic effects of this drug along with the highest tolerability and safety were examined in various experimental models such as nephritic syndrome, experimental autoimmune encephalomyelitis (EAE), adjuvant-induced arthritis (AIA), immune complex glomerulonephritis (ICG) and BC murine model.¹⁹⁻²¹

The importance of inflammation in tumor progression comes from the effectiveness of long-term and regular use of non-steroidal anti-inflammatory drug (NSAIDs), such as ibuprofen and aspirin in inhibition and treatment of colorectal, breast and other cancers.²² The use of NSAIDs are related to 10–20% reduced risk of BC and some epidemiologic studies have reported that this protective effect of NSAIDs on BC risk could be more strong for hormone receptor-positive tumors.^{23,24} However, long-term use of NSAIDs for therapeutic purposes is associated with GI, cardiovascular and renal toxicities which limit their use.²⁵ Hence, it is worthwhile to investigate for alternative anti-inflammatory drugs that are safe for long-term use. It has been proven that both in situ and invasive human breast tumor cells overexpress Cyclooxygenase-2 (COX-2). NSAIDs and novel agent M2000 have shown to have relevant anti-cancer activity by blocking the COX-2 signaling pathway.²⁶

The present research was conducted to evaluate the effects of M2000 on the levels of MMP-2, MMP-9, CCL22 and TGF β 1 gene expression and Tregs frequency, in BC patients.

MATERIALS AND METHODS

Study Design and Patients

This study was a phase II, randomized, controlled clinical trial (clinical trial identifier: IRCT2017012213739N7) between Iranian women with invasive ductal carcinoma BC. This trial was designed to evaluate the effects of M2000 on MMP-2, MMP-9, CCL22, and TGF β 1 gene expression and Tregs frequency during 6-8 weeks in pre-surgical BC patients. The study design and investigation was approved by the ethics committee of Tehran University of Medical Sciences (N.: IR.TUMS.SPH.REC.1395.1490). The present research was done at the Breast Cancer Research Center (Imam Khomeini Hospital, Tehran, Iran) in accordance with the declaration of Helsinki. The written informed consent was obtained from all participants.

A total of 24 women patients were included in the study so that 12 patients were randomly assigned to assess the effect of M2000 on MMP-2, MMP-9, CCL22, and TGF β 1 gene expression and Tregs frequency were tested. The exclusion criteria were a history of fever and infectious diseases, positive pregnancy test, history of other concomitant (hepatic, renal, cardiovascular, autoimmune, neurological, psychiatric, bleeding and endocrinologic) diseases. Treatment group that was on the waiting list for surgery were followed with fixed oral doses of M2000, 500 mg two times a day (6-8 weeks). Blood samples were collected at baseline and weeks 6-8 (one day before surgery). To compare the patterns of MMP-2, MMP-9, CCL22, and TGF β 1 gene expression and Tregs frequency of healthy women normal controls with BC patients, a set of 10 blood samples of women healthy volunteers were collected at the Iran Blood Transfusion Organization (Tehran, Iran).

RNA Isolation and cDNA Synthesis

The blood samples of BC patients and healthy controls (6 mL) were collected into EDTA containing tube (BD Vacutainer, Plymouth, UK) by venipuncture. The peripheral blood mononuclear cell (PBMCs) was isolated from freshly obtained blood by Ficoll density gradient centrifugation (Lymphosep, Biosera, Paris, France) and was immediately processed for the total mRNA extraction of MMP-2, MMP-9, CCL22 and TGF β 1 genes using total RNA purification kit (Hybrid RTM GeneAll, Seoul, Korea) according to the

manufacturer's protocol. The integrity and concentration of total RNA were quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Utah, USA) and the samples with OD260/280 ratio in the range of 1.7–2.0 were used for complementary DNA synthesis. The first-strand cDNA was synthesized via oligo-dT and random 6-mer, primers in the presence of reverse transcriptase using PrimeScript TM RT reagent Kit (Takara, Shiga, Japan).

Quantitative Real-time PCR (qRT-PCR)

The mRNA levels of targeted MMP-2, MMP-9, CCL22, and TGF β 1 genes were assayed using Syber premix Ex TaqTM II (Takara, Shiga, Japan) in ABI Step One PlusTM Real-Time PCR System (Thermo Scientific, USA). All samples were measured in triplicate, and the reactions' volume was 25 μ L containing the components as listed: 2 μ L of cDNA, 12.5 μ L of SYBR Green master mix and 1 μ L of forward and reverse primers and 8.5 μ L of dH₂O sterile distilled water. The PCR was conducted as follows: the first denaturation at 95 °C for 30 seconds followed by 40 cycles of denaturation at 95°C for 5 seconds, annealing at 60 °C for 30 seconds. Real-time PCR was conducted using gene-specific primers as listed in (Table 1). The relative MMP-2, MMP-9, CCL22, and TGF β 1 mRNA levels were normalized to the endogenous control GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Finally, mean fold changes were calculated by the $2^{-\Delta\Delta CT}$ method to analyze the relative quantitation of MMP-2, MMP-9, CCL22, and TGF β 1 genes.

Flowcytometric Analysis

The blood samples of BC patients and healthy controls (4 mL) were collected into EDTA containing tube (BD Vacutainer, Plymouth, UK) by venipuncture. The PBMCs were obtained from both patients and healthy controls using Lymphocyte Separation Media (Lymphosep, Biosera, France) and re-suspended in the RPMI medium (Lymphosep, Biosera) supplemented with 10% fetal bovine serum (Lymphosep, Biosera), penicillin (100 IU), and streptomycin (100 μ g/mL) (Biosera, Ringmer, East Sussex, UK) for Tregs isolation. To evaluate Tregs frequency, PBMCs were stained using surface antibodies (anti-CD4 PerCP-cy5.5, anti-CD25 APC [clone BC96] and anti-CD127 FITC [clone eBioRDR5], eBioscience) and were incubated in the dark at 4°C for 30 minutes.

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Table 1. Primer sequences for evaluation of gene expression in peripheral blood mononuclear cells of breast cancer patients

No.	Gene name	Accession number	Primer Sequences
			5' → 3'
1	GAPDH	NM_001289746.1	5'-GAGAAGGCTGGGGCTCATTT-3' 5'-TAAGCAGTTGGTGGTGCAGG -3'
2	MMP-2	NM_004530	5'-TACAGGATCATTGGCTACACACC-3' 5'-GGTCACATCGCTCCAGACT -3'
3	MMP-9	NM_004994.2	5'-TACAGGATCATTGGCTACACACC -3' 5'-GGTCACATCGCTCCAGACT-3'
4	CCL22	NM_002990.4	5'-ATTACGTCGGTTACCGTCTGC-3' 5'-TCCCTGAAGGTTAGCAACACC-3'
5	TGFβ1	NM_000660.6	5'-CAATTCCTGGCGATACCTCAG-3' 5'-AGATAACCACTCTGGCGAGTC-3'

BC: breast cancer; PBMCs: peripheral blood mononuclear cells; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; MMP-2: matrix metalloproteinase-2; MMP-9: matrix metalloproteinase-9; CCL22: C-C motif chemokine 22; TGFβ1: transforming growth factor beta 1

Following surface staining, the cells were washed twice, fixed and permeabilized with Fix/Perm buffer and suspended in permeabilization buffer (eBioscience). Then the anti-FOXP3 PE [Forkhead Box P3, clone 236A/E7] for evaluation of Tregs, were added and incubated at room temperature for 30 min. Cells were washed with permeabilization buffer, re-suspended in cold staining buffer, and determined by BD FACS Calibur Flow Cytometer (BD Biosciences, USA). Lymphocytes were gated on forward and side scatters' profiles and analyzed using FlowJo software (Tree Star, USA). Isotype-matched control antibodies and fluorescence minus one (FMO) control stains were used to determine background levels of staining.

Statistical Analysis

All results were expressed as mean±standard error of means (SEM) for numerical variables and frequencies and percentages for categorical variables. Treatment group differences in continuous variables were compared with analysis of covariance, with baseline values as covariance. The MMP-2, MMP-9, CCL22, and TGFβ1 gene expression and Tregs frequency (percentage) before and after treatment with M2000 were tested using paired Student's T-tests and the standard analysis of variance (ANOVA) was used for comparing the results of the normal group with treatment groups. When the significant effects were found, Tukey post hoc multiple comparison testing was used for further analysis between control and treatment groups. All statistical data were two-sided and analyzed

using the SPSS software version 24 (IBM Corporation, Armonk, NY, USA) and *P* values of less than 0.05 were considered statistically significant.

RESULTS

Effect of M2000 on MMP-2, MMP-9, CCL22 and TGFβ1 Gene Expression in BC Patients

Our data indicated that the level of MMP-2 gene expression in BC patients (6.37±2.58-fold) was higher than healthy controls (1.18±0.34-fold) (*p*=0.08). The results also demonstrated that the fold change of MMP-2 after 6-8 weeks treatment with M2000 reduced to (1.49±0.36-fold) but the difference of MMP-2 gene expression before and after treatment with M2000 was not significant (*p*=0.08) (Figure 1). Our complementary data showed that the level of MMP-9 gene expression in BC patients increased up to (4.43±0.95-fold) in comparison with healthy controls (0.77±0.26-fold) (*p*=0.05). After 6-8 weeks, we evaluated the MMP-9 gene expression. Results showed that the gene expression decreased (1.95±0.47-fold) and the difference in fold changes between patients before and after M2000 therapy was statistically significant (*p*=0.03) (Figure 2).

The level of CCL22 gene expression in BC patients increased up to (2.81±0.68-fold) in comparison with healthy controls (0.64±0.25-fold) (*p*=0.01). After 6-8 weeks, we evaluated the CCL22 gene expression. The results revealed that the gene expression decreased to (0.63±0.14- fold) and the difference in fold changes

between patients before and after M2000 therapy was significant ($p=0.003$) (Figure 3).

The level of TGF β 1 gene expression in BC patients increased up to (1.75 ± 0.2 -fold) in comparison with healthy controls (1 ± 0.1 -fold) ($p=0.03$). After 6-8

weeks, we evaluated the TGF β 1 gene expression. The results revealed that the gene expression decreased to (1.22 ± 0.19 - fold) but the difference in fold changes between patients before and after M2000 therapy was not statistically significant ($p=0.1$) (Figure 4).

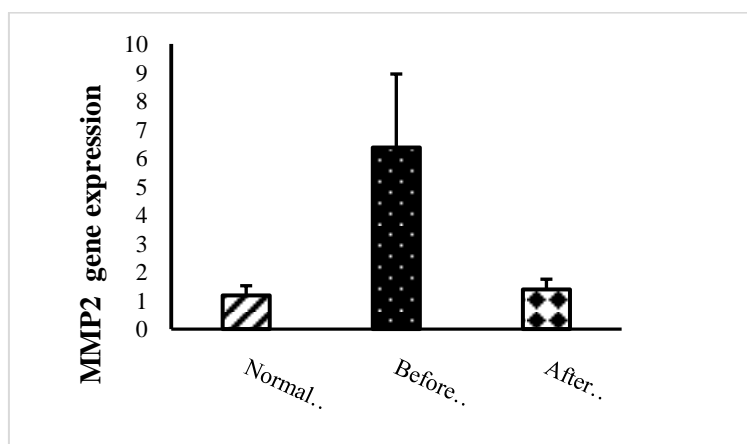


Figure 1. The effect of M2000 on MMP-2 gene expression in breast cancer patients and healthy controls PBMCs. The groups were healthy control, before treating patients and after treating patients with M2000 (1000 mg/day). The MMP-2 gene expression was measured by qRT-PCR. Relative quantification of gene expression was obtained by the $2^{-\Delta\Delta CT}$ methods. Values were normalized by GAPDH. Data are presented as mean \pm SEM.

M2000; β -D-Mannuronic, MMP-2; matrix metalloproteinase-2, BC; breast cancer, PBMCs; peripheral blood mononuclear cells, qRT-PCR; quantitative real-time PCR, GAPDH; glyceraldehyde-3-phosphate dehydrogenase

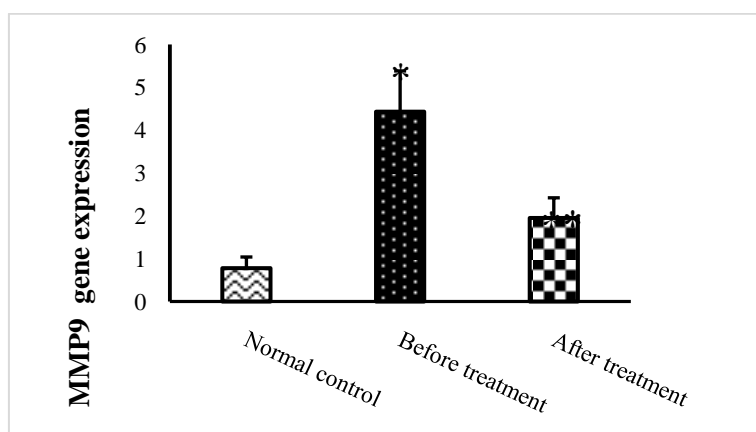


Figure 2. The effect of M2000 on MMP-9 gene expression in breast cancer patients and healthy controls PBMCs. The groups were healthy control, before treating patients and after treating patients with M2000 (1000 mg/day). The MMP-9 gene expression was measured by qRT-PCR. Relative quantification of gene expression was obtained by the $2^{-\Delta\Delta CT}$ methods. Values were normalized by GAPDH. Data are presented as mean \pm SEM. *=0.050 vs. healthy controls, **=0.03 vs. before treatment patients.

M2000; β -D-Mannuronic, MMP-9; matrix metalloproteinase-9, BC; breast cancer, PBMCs; peripheral blood mononuclear cells, qRT-PCR; quantitative real-time PCR, GAPDH; glyceraldehyde-3-phosphate dehydrogenase

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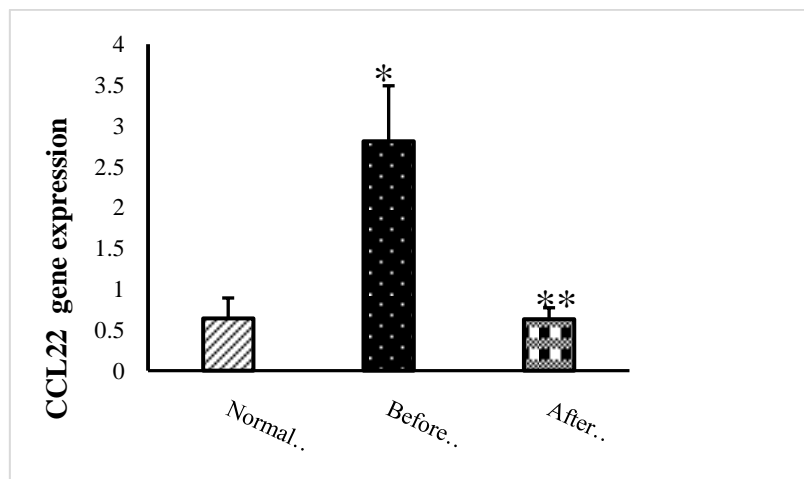


Figure 3. The effect of M2000 on CCL22 gene expression in breast cancer patients and healthy controls PBMCs. The groups were healthy control, before treating patients and after treating patients with M2000 (1000 mg/day). The CCL22 gene expression was measured by qRT-PCR. Relative quantification of gene expression was obtained by the $2^{-\Delta\Delta CT}$ methods. Values were normalized by GAPDH. Data are presented as mean \pm SEM. *=0.01 vs. healthy controls, **=0.003 vs. before treatment patients.

M2000; β -D-Mannuronic, CCL22; C-C motif chemokine 22, BC; breast cancer, PBMCs; peripheral blood mononuclear cells, qRT-PCR; quantitative real-time PCR, GAPDH; glyceraldehyde-3-phosphate dehydrogenase

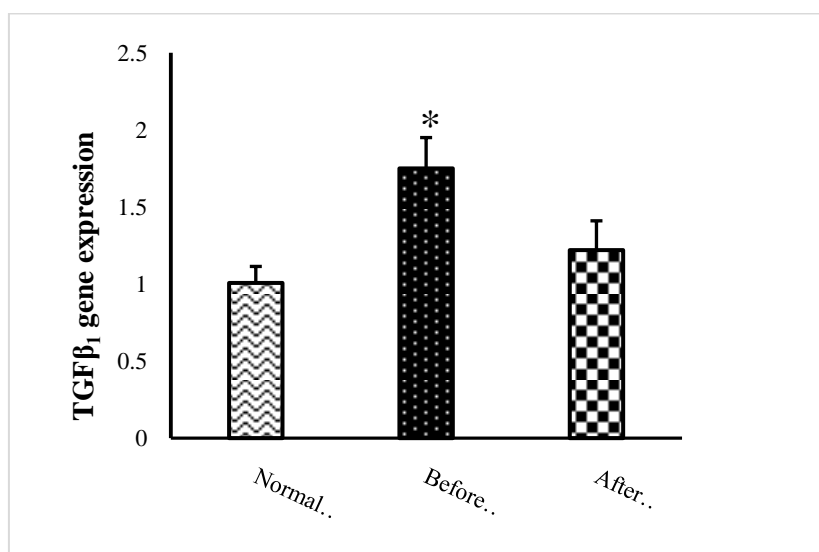


Figure 4. The effect of M2000 on TGFβ1 gene expression in breast cancer patients and healthy controls PBMCs. The groups were healthy control, before treating patients and after treating patients with M2000 (1000 mg/day). The TGFβ1 gene expression was measured by qRT-PCR. Relative quantification of gene expression was obtained by the $2^{-\Delta\Delta CT}$ methods. Values were normalized by GAPDH. Data are presented as mean \pm SEM. * $p=0.030$ vs. healthy controls.

M2000; β -D-Mannuronic, TGFβ1; transforming growth factor beta 1, BC; breast cancer, PBMCs; peripheral blood mononuclear cells, qRT-PCR; quantitative real-time PCR, GAPDH; glyceraldehyde-3-phosphate dehydrogenase

Effect of M2000 on Tregs Frequency in BC patients

The frequency of Tregs (CD4⁺, CD25⁺, FoxP3⁺, CD127^{low/-}) in PBMCs of BC patients and healthy controls were evaluated. The baseline frequency of circulating lymphocyte cells in BC patients (60.6%±7.1%) was less than healthy controls (70%±5.4%) ($p=0.5$). After 6-8 weeks of M2000 therapy, the frequency of circulating lymphocyte cells in BC patients (62.2%±3.3%), was higher than patients before M2000 therapy ($p=0.9$) (Figure 5). Also, we found that the frequency of circulating T CD4⁺ cells in BC patients (25.6%±7.6%) was less than healthy

controls (47.7%±2%) ($p=0.04$) which was statistically significant. After 6-8 weeks of M2000 therapy, the frequency of circulating T CD4⁺ lymphocyte cells in BC patients (41.7%±5.7%), was higher than patients before therapy ($p=0.8$) (Figure 6). The frequency of circulating Tregs (CD4⁺, CD25⁺, FOXP3⁺, CD127^{low/-}) in BC patients (1.99%±0.22%) was higher than healthy controls (1.63%±0.39%) ($p=0.6$). After 6-8 weeks of therapy with M2000, the frequency of circulating Tregs in BC patients (1.38%±0.19%), was less than patients before therapy which was statistically significant ($p=0.01$) (Figure 7).

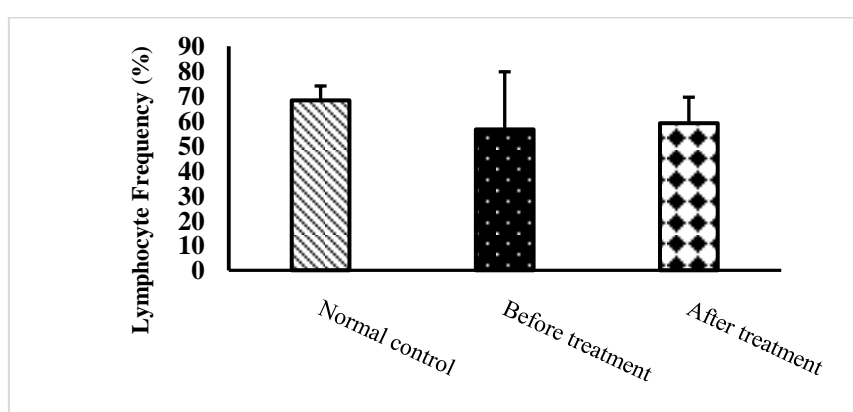


Figure 5. The effect of M2000 on lymphocytes frequency in breast cancer patients and healthy controls PBMCs. The groups were healthy control, before treating patients and after treating patients with M2000 (1000 mg/day). The lymphocytes frequency was measured by flow cytometry. Lymphocytes were separated by side scatter and forward scatter. Bars show the mean values±SEM.

M2000; β-D-Mannuronic, BC; breast cancer, PBMCs; peripheral blood mononuclear cells

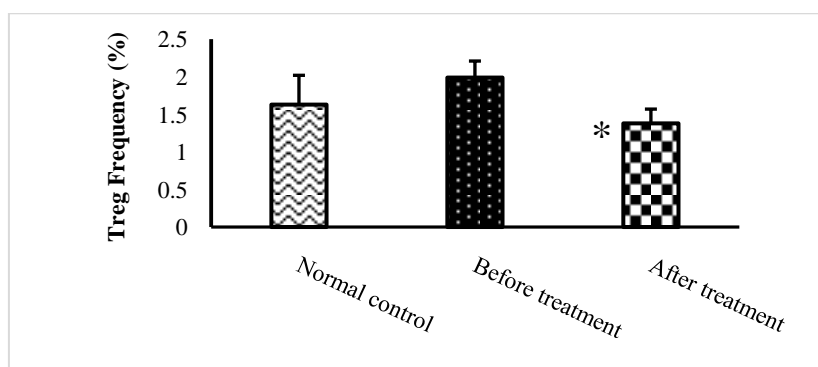


Figure 6. The effect of M2000 on TCD4⁺ lymphocytes frequency in breast cancer patients and healthy controls PBMCs. The groups were healthy control, before treating patients and after treating patients with M2000 (1000 mg/day). The TCD4⁺ lymphocyte frequency was measured by flow cytometry. T CD4⁺ lymphocytes were separated by Anti-Human CD4 PerCP-cyanine 5.5 IgG1, k. Bars show the mean values ± SEM. *=0.04 vs. healthy controls.

M2000; β-D-Mannuronic, PBMCs; peripheral blood mononuclear cells

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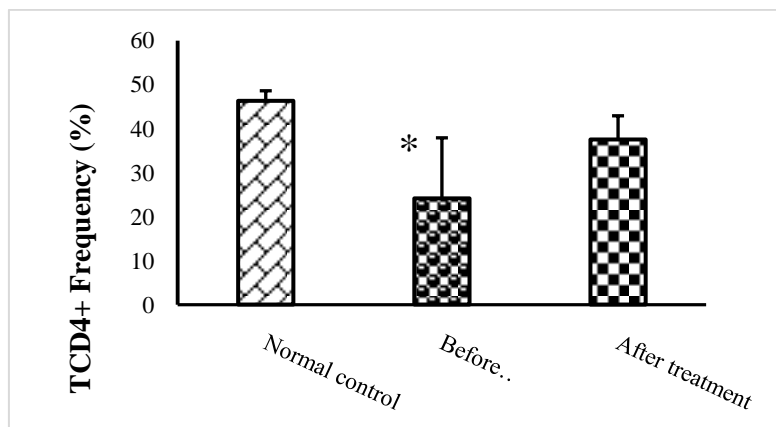


Figure 7. The effect of M2000 on Tregs frequency in breast cancer patients and healthy controls PBMCs. The groups were healthy control, before treating patients and after treating patients with M2000 (1000 mg/day). The Tregs frequency was measured by flow cytometry. Tregs were separated by Anti-Human CD4 PerCP-cyanine 5.5 IgG1, k; Anti-Human CD25 APC IgG1, k; Anti-Human FOXP3 PE IgG1, k and Anti-Human CD127 FITC IgG1, k. Bars show the mean values \pm SEM. * ≤ 0.01 vs. before treating patients.

M2000; β -D-Mannuronic, Tregs; T regulatory Cells, PBMCs; peripheral blood mononuclear cells, FOXP3; forkhead box P3, PE; phycoerythrin, FITC; Fluorescein isothiocyanate

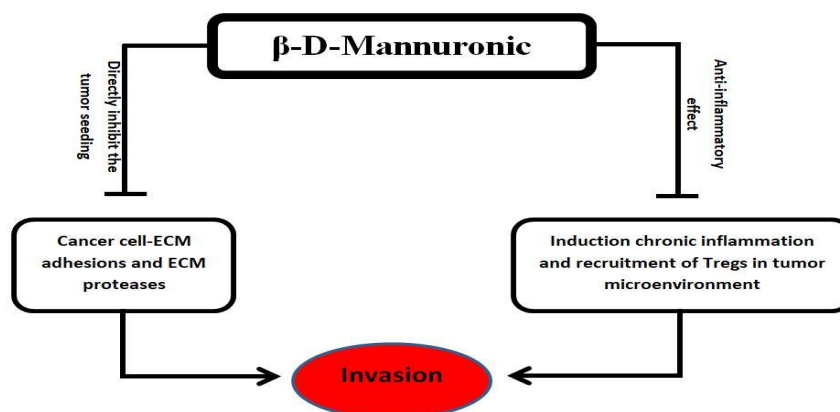


Figure 8. Schematic hypothesis: the anti-cancer effect of β -D-mannuronic acid (M2000)

DISCUSSION

Some data show that inflammatory responses following crosstalk between tumor cells and surrounding microenvironment are vital for Tumor development.²² TILs correlated to axillary lymph node status, tumor diameter and histomorphological variables. The inflammatory mediators could affect angiogenesis by the release of angiogenic factors and digesting matrix. Angiogenesis is an essential step in BC progression and the density of the vessels is an

important prognostic factor for relapse and overall survival. previous studies showed that M2000 has an anti-angiogenesis and anti-proliferative effects in-vitro and in-vivo models.²⁷

The Tregs level in the peripheral blood of BC patients has been found to be high. This high Tregs level has a correlation with poor prognosis. It has been reported that BC patients with high MMP-2, MMP-9, CCL22, and TGF β 1 gene expression have shown tumor progression, metastasis, angiogenesis, and poor prognostic outcome. So, factors that regulate tumor

interaction are an attractive target for therapeutic applications.²⁸⁻³⁰ The BC murine model demonstrated that M2000 strongly inhibits tumor growth and increases lifespan compared with control mice. The decrease in tumor mass was associated with a decrease in the recruitment of inflammatory cells in the tumor microenvironment and prevented tumor metastasis.²² Furthermore, the significant reduction in Tregs frequency and chemotactic factor has been observed in M2000 treated group. MMPs have a main role in the extracellular matrix (ECM) destruction and tumor cells migration. previous research showed that M2000 inhibits MMP-2 and MMP-9 activity in WEHI-164 and THP-1 cell line.³¹ some data suggested that M2000 can induce apoptosis in cultured fibrosarcoma cells. M2000 can induce programmed cell death in cancer cell lines as a novel NSAID.³²

It has been proven that both in situ and invasive human breast tumor cells overexpress COX-2. The novel agent M2000 has anti-cancer and anti-inflammatory activity by blocking the COX-2 signaling pathway and PGE2 production.³³

M2000 causes chronic inflammation reduction by decrease Tregs frequency and CCL22 production from TILs and tumor cells that they have the main role in tumor suppression. CCL22 is a main chemotactic factor for Tregs and other TILs.²²

The Mannose receptors (MRs) are essential components in cancer cell adhesion and cell traffic within the lymphatic system and blood circulation. As proof of concept, several mannose-based cancer therapy approaches have been reported.³⁴ It seems that M2000 with a low molecular weight (<200 Dalton) and its mannosyl structure bind to MRs family. However, further investigation on whether M2000 regulates anti-tumoral response based on MR mechanism is needed.

This study was done on BC patients for the first time and patients were waiting for surgery. The ethics committee of Tehran University of Medical Sciences approved only 24 patients. Also, taking blood samples more than 10 mL was against the roles of the ethics committee. There are some limitations in this study that could be addressed in future research. First, the study focused on a small sample size. Short-term M2000 therapy was another limitation. If a larger sample size and long-term M2000 therapy is approved by the Ethics Committee with, we will have a better evaluation of the effects of this new NSAID.

In this study, we investigated the anti-tumoral effect

of M2000 on BC and we showed that M2000 has anti chronic inflammatory, high safety and inhibitory effects against MMP-2, MMP-9, CCL22 and TGFβ1 gene expression and Tregs frequency in BC (Figure 8). This study is the first evidence of therapeutic effects of M2000 in BC patients and supports the role of anti-inflammatory agents in cancer therapy. Further study may help to find more anti-tumoral effects of M2000 by evaluating more patients and more inflammatory mediators in BC. Our data suggested that M2000 can reduce gene expression of MMP-2, MMP-9, CCL22 and TGFβ1, and Tregs frequency. Therefore M2000 could be a suitable agent in preventing chronic inflammatory reactions, angiogenesis, and metastasis without adverse effects. There is therapeutic potency of M2000, as a novel designed NSAID, for patients with BC. Our data suggested that we can use other treatments for BC except, standard and well-known treatments.

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