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Research Article

Effects of M2000 and Vitamin D3 in the Expression of IL-10, IFN-γ and IL-6 in Crohn's Diseases

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Abstract

Background: Crohn's disease is a chronic, transmural immune-mediated inflammatory disease of the gastrointestinal tract (GIT). It can attack any portion of the GIT, although inflammation most commonly occurs in the lower portion of the small intestine, known as the *ileum*. The disease can cause ulcerations within the intestine, which is capable of eroding into surrounding tissues, such as the bladder, vagina and the surface of the skin. Inflammation in Crohn's disease is not limited to the intestine, some Crohn's disease patients have inflammation of the eyes and joints as well. This research aimed to assess the efficacy of M2000 and vitamin D3 in the treatment of Crohn's disease.

Materials and Methods: Fifteen (15) ml of blood was collected from 40 research participants. The PBMC was isolated, then stimulated with LPS and incubated for 4 hours. The cells were later treated with various doses of M2000 and vitamin D3 and incubated for 24 hours at optimum condition. The cells were harvested and centrifuged at 12,000g for 15 minutes. RNA was extracted and converted to cDNA. Real-time PCR and ELISA were used for gene and cytokine expressions, respectively.

Results: The baseline expressions of IL-6 and IFN-γ were significantly upregulated in the positive controls in contrast to IL-10. After treatment, significant down-regulation in IL-6, and IFN-γ was observed, while IL-10, was significantly up-regulated.

Conclusion: The result indicated that M2000 and vitamin D3 are potent immunosuppressive and immunomodulatory agents, that may be used in Crohn's disease management. It recommended that further researches and clinical trials should be initiated to ascertain the place of vitamin D3 and M2000, co-administration in Crohn's disease management.

Keywords: Vitamin D3, IL-6, IFN-γ, IL-10, M2000, Crohn's disease

Abbreviations: APC: Antigen presenting cells; ASCA: Anti-Saccharomyces cerevisiae antibodies; Avidin-HRP: Avidin horseradish peroxidase; B-regs cells : B-regulatory cells; CD: Crohn's diseases; CD 4+ : T-helper cells; CD 8+ : Cytotoxic T cells; cDNA : Complimentary DNA; CRP: C-reactive protein; DC: Dendritic cells; DDRI: Digestive Disease Research Institute; ELISA: Enzyme linked immunosorbent Assay; ESR: Erythrocyte sedimentation rate; FBS: Fetal bovine serum; FOXP3: Forkhead box P3; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; GIT: Gastrointestinal tract; HHDMVD: Half high dose Mannuronic acid + Vitamin D3; HLDMVD: Half low dose Mannuronic acid + Vitamin D3; IBD: Inflammatory bowel disease; IFN-γ : Interferon gamma; IL-6: Interleukin- 6; IL-10: Interleukin-10; IL-12: Interleukin-12; LPS : Lipopolysaccharides; M2000: β-D-mannuronic acid; MAPK: Mitogen-activated protein kinase; MHD: Mannuronic acid high dose; MKP-1 : MAPK phosphatase-1; MLD: Mannuronic acid low dose; MS : Multiple sclerosis; NC: Normal control; NF-κB : Nuclear factor κappa Beta; NSAIDs: Non steroidal anti-inflammatory drugs; P-ANCA : Perinuclear anti-neutrophil cytoplasmic; PBMC : Peripheral blood mononuclear cells; PC: Positive control; PCR: Polymerase chain reaction; RA: Rheumatoid arthritis; rHuIL-10: Recombinant IL-10; RPMI : Roswell Park Memorial Institute medium; RNA : Reoxyribonucleic acid; STAT3: Signal transducer and activator of transcription-3; TCRs: T cells receptors; TGF-β : Transforming growth factor Beta; Th1: T helper-1 cells; TLRs : Toll like receptor; TMB : Tetramethylbenzidine; TNF-α : Tissue necrosis factor alpha; Treg : T regulatory cells; UC : Ulcerative colitis; VDRs: Vitamin D receptor; VHD/VLD: Vitamin D3 high dose/Vitamin D3 low dose

Introduction

Crohn's disease is a chronic, transmural immune-mediated inflammatory disease of the GIT. It can attack any portion of the digestive tract, although inflammation most commonly occurs in the lower portion of the small intestine, known as the *ileum*. The disease can cause ulcerations within the intestine that can erode into surrounding tissues such as the bladder [1], vagina [2], or even the surface of the skin [3]. Inflammation in Crohn's disease is not limited to the intestine—some people who have Crohn's disease have inflammation of the eyes and joints as well [3]. The most common symptoms of the disease include severe abdominal pain with or without diarrhoea. Diarrheal stool may be mixed with blood, mucus and/or pus. Bowel movements are often painful. Cramping in the right lower side of the abdomen is common, especially after meals. Crohn's disease patients have a characteristic chronic low-grade fever, poor appetite, fatigue, weight loss, Skin rashes [3], and some degree of anaemia, related to poor iron, folic acid, and vitamin B12 absorption, due to chronic blood loss.

Diagnosis of Crohn's disease is usually based on a patient's medical history and symptoms. Diagnostic tests may be used to confirm the disease and to distinguish it from ulcerative colitis. Such tests include x-rays, colonoscopy, and endoscopy [4]. No blood test can diagnose Crohn's disease, but routine testing is usually done to detect anaemia, infection, degree of inflammation, and determine liver function [4]. Certain markers of inflammation, such as erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) may be used to follow a patient's course over time. The anti-*Saccharomyces cerevisiae* antibody (ASCA) blood test is sometimes used to help differentiate Crohn's disease from ulcerative colitis [4].

IL-10 is an anti-inflammatory cytokine that inhibits both antigen presentation and subsequent release of pro-inflammatory cytokines, thereby attenuating mucosal inflammation. The pivotal role played by IL-10 within the mucosal immune system has

been extensively studied in the chronic ileocolitis that develops in gene-targeted IL-10 knockout mice and by its therapeutic efficacy in several animal models of colitis [5]. An inactivation of IL-10 in mice results in an increased production of IL-12 and IFN-γ [6]. Inflamed tissues and granulomas of CD show low IL-10 [7]. Melgar *et al* reported a highly significant increase in IL-10 mRNA levels in T lymphocytes and IL-10-positive cells in the colons of UC patients [8]. Recently, the production of IL-10 by regulatory T cells has been implicated as an important factor in IBD [9]. Other regulatory cells that may participate in UC through the production of IL-10 are a regulatory B cells subtype called Bregs [10]. The importance of IL-10 production by B cells has been evidenced in IBD models and humans [11], Mizoguchi *et al* showed that B-regs can be responsible for the suppression and/or recovery from acquired immune-mediated inflammations by mechanisms that include IL-10 and TGF-β1 in IBD [10].

The IL---6 / STAT3 signalling system plays an important role in CD pathogenesis. Circulating levels of IL-6 and s-IL-6R correlate with disease activity [12]. The pathogenic role of the IL-6 and s-IL-6R signalling in interfering with T-cell resistance to CD apoptosis was confirmed by blocking IL-6 trans-signaling[13]. These research findings have shown that IL-6 can influence not only the chronic inflammatory pathways but also the relapses that arise in the pathology of CD [14]. The effects of IL-6 in IBD were demonstrated in Caco2 cells, where it was shown that IL-6 induces the activation of NF-kappa β and enhances the expression of intercellular adhesion 1 molecule [15].

It is well-established that vitamin D plays a critical role in improving bone health and is specifically important for patients who are at risk of low bone density. Low bone mineral density is more prevalent among patients with Crohn's disease and ulcerative colitis compared to healthy controls [16]. There is, however, growing support for non-traditional actions of vitamin D including anti-inflammatory, antiproliferative, cell differentiation, and apoptotic effects. These effects have led to the examination of vitamin D in the pathogenesis of autoimmune diseases, such as in IBD, with special emphasis on Crohn's disease [17].

M2000 is a copolymer of sodium alginate, which has so far demonstrated its enormous immunomodulatory potentials in various experimental models of autoimmune diseases [18], such as in nephrotic syndrome, multiple sclerosis (MS), immune complex glomerulonephritis and Rheumatoid arthritis (RA) [19,20].

There is a need to develop a safe and effective drug for the management of this disease, this is because most of the drugs used in the treatment have not yielded the desirable outcomes. This research was designed to test the therapeutics effects of M2000 and Vitamin D3 on IL-10, IL-6, and IFN-γ expression in Crohn's disease patients.

Materials and methods

Study population

A total number of 40 volunteers consisting of 20 Crohn's disease patients and 20 healthy controls was recruited for this research. These include newly diagnosed patients that are not, on any immunomodulatory agents. Smokers, pregnant and lactating mothers, drug users and HIV positive persons, were all excluded from participating in the research. The ethical approval for the conduct of this research was obtained from the ethical committee of Tehran University of Medical Sciences (TUMS), Tehran. Written and signed informed consent, was obtained from all the research participants. The research was conducted in line with the international best practice in conformity with the international standard ethical protocol.

Location of the study

This research was carried out in the Department of Pathobiology/ Immunology, Tehran University of Medical Sciences, International campus (IC-TUMS), Islamic Republic of Iran(IRI).

Research group.

The research is made up of eight (8) groups, each matched with appropriate normal controls. These are: Normal control (NC), Positive control(PC), Mannuronic acid low dose(MLD), Mannuronic acid high dose(MHD), Vitamin D3 low dose(VLD), Vitamin D3 high dose (VHD), Half low dose Mannuronic+Vitamin-D3 (HLDMVD) and Half high dose Mannuronic+Vitamin D3 (HHDMVD)

Sample collection

Fifteen (15) ml of blood was aseptically collected from the research participants into an EDTA bottle, the bottles were gently mixed and instantly labelled for proper identification.

Reagents and kits

All the kits and reagents used in this research are of international grade. Some of the reagents/kits used are:

- LPS- sigma Aldrich Germany
- Ficol-paque reagents- Amersham Pharmacia Biotech, Uppsala-Sweden
- RPMI 1640 (GIBCO) with 10% FBS (GIBCO) and 1% pen/strep
- GeneAll® Hybrid-RTM kits Cat. No. 305-101 (Songpa-Gu, Seoul, Korea 138-859).
- cDNA prime-script TM reagent Kit, Takara BIO. INC (Perfect Real Time), Cat NO: RR037A, lot NO: AK5601 (Nojihigashi 7-4-38, Kusatsu, Shiga 525-0058 Japan)
- Specific Human IFN-γ ELISA Ready-Set-Go kitR, catalogue number: 88-7316 and
- Specific Human IL-6 ELISA Ready-set-Go kitR, catalogue number: 88-7066 (EBioscience, Inc, an Affymetrix).
- SYBR® Premix Ex Taq™ II (Takara Co., Ltd.) Japan
- Specific IL-10 and GAPDH primers (Sigma-Aldrich) Germany

PBMC Isolation

The PBMC isolation was performed using the Ficol-Paque centrifugation method (Amersham Pharmacia Biotech, Uppsala-Sweden). The PBMC was isolated in strict compliance with the manufacturer's instructions. After the PBMC was isolated, 3ml of complete culture medium containing RPMI 1640 (GIBCO) with 10% FBS (GIBCO) and 1% pen/strep was added to the PBMC and carefully homogenised in the medium and counted using an improved Neubauer haemocytometer counting chamber.

Cell culture

In a 24-well culture plate, 2.0 x106 PBMCs were seeded in each well. The cells were stimulated

with 1μg/ml of LPS (Sigma-Aldrich) and incubated for 4 hours at 370C, in 5% CO2 and 100% humidified air. After incubation, the PBMCs were appropriately treated with 10μg/ml and 50μg/ml of low and high doses of M2000, 10-10M and 10-8M of low and high doses of Vitamin D3 and half low doses of M2000 plus Vitamin D3 (5μg/ml+5 X10-11M) and half high doses of Mannuronic acid plus Vitamin D3 (25μg/ml+5x10-9M) respectively, and then incubated for an additional 48 hours at the same culture conditions.

RNA extraction

The PBMCs were harvested from the cell culture plates into a 2ml Eppendorf tube and centrifuge at 12,000 RCF for 10 minutes to separate the cells from the supernatants. The supernatants were stored at -700C until ready for cytokines assay. Total RNA was extracted from 2.5×106– 3×106 cells, using GeneAll® Hybrid-RTM kits Cat. No. 305-101 (Songpa-Gu, Seoul, Korea 138-859).

The cDNA synthesis

The synthesis of the cDNA was performed using the cDNA prime-script reagent Kit, Takara BIO. INC (Perfect Real Time), Cat NO: RR037A, lot NO: AK5601 (Nojihigashi 7-4-38, Kusatsu, Shiga 525-0058 Japan), based on the manufacturer's instructions, the cDNA was synthesized.

ELISA for evaluation of IFN-γ and IL-6

The concentrations of IFN-γ and IL-6 in supernatants from 48-hours PBMCs cultures were determined, using specific Human IFN-γ ELISA Ready-Set-Go kitR, catalogue number: 88-7316 and specific Human IL-6 ELISA Ready-set-Go kitR, catalogue number: 88-7066 (EBioscience, Inc, an Affymetrix). This evaluation was carefully carried out in line with the manufacturer's instructions.

Real-Time-PCR

The Real-time PCR was performed using SYBR® Premix Ex Taq™ II (Takara Co., Ltd.) with all the specific primers (Sigma-Aldrich) (Table 1), based on the provided guidelines. The gene transcrip-

Table 1: Primer sequences for Real-time PCR

Primer	Forward Sequence	Reverse Sequence
IL -10	CGAGATGCCTTCAGCAGAGT	CGCCTTGATGTCTGGGTCTT
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

tion analysis of IL-10, and GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) as the housekeeping gene, was performed using StepOnePlus™ Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA). The relative expressions of target interleukin mRNA compared against the endogenous gene, GAPDH mRNA, were measured using a ΔCT method regarding each amplification plot (fluorescence signal vs cycle number). The mean difference (ΔCT) between the values in the replicate samples of target cytokine and that of the endogenous control, GAPDH mRNA was calculated. The changes in the expressions of the target cytokine and the normal controls were calculated using, ΔCTPatients – ΔCTControls = ΔΔCT. This is further expressed as a relative fold change or gene expression in the patients compared to the normal and healthy control (2^-ΔΔCT).

Statistical Analysis

SPSS software (22.0; IBM Corporation, Chicago, IL, USA) was used to carry out all the statistical analyses. The mean standard deviation was used to express all the data and the P-value of (P<0.05) was statistically considered as a significant expression of all the genes. The normality of all the data was checked by the use of the Kolmogorov-Smirnov test. The comparison of the quantitative variables between the groups was done by the means of the Analysis of Variance (ANOVA) test and Post hoc Turkey to determine significant differences in the gene expression level between the treated and the untreated groups. The design of all the graphs was done by the use of GraphPad Prism software version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

The roles of M2000 and Vitamin D3 has been quite elucidated by these research findings. Both the drugs have expressed their immunosuppressive and anti-inflammatory potentials

Effects of M2000, and Vitamin D3 on IL-10 Expression

The baseline fold changes in the positive controls for IL-10 was 0.3 fold, but after the treatment with low and high doses of M2000, the relative expressions of IL-10 were upregulated to 1.10 (P< 0.01) and 2.7(P<0.0001) fold respectively. After treatment with Vitamin D3, the relative expression of IL-10 was upregulated to 0.90(P< 0.01) and 2.05(P<0.001) fold for low and high doses of Vitamin D3 respectively. When the PBMC was treated with half low and half high doses of M2000 and Vitamin D3, the relative expression of IL-10 was upregulated to 0.97 (P<0.01) and 2.49(P<0.0001) fold respectively. This represents significant up-regulation, compared with the positive controls. The P < 0.05 was considered statistically significant (Figure1)

Effects of M2000, and Vitamin D3 on IFN-γ expression

The baseline expression of IFN-γ in the PBMCs of the normal and positive controls was 11.1pg/ml and 30.7 pg/ml, respectively. After treatment and 24 hours of incubation of patients' PBMCs with low and high doses of M2000, the levels of IFN-γ cytokine expression in the treated PBMCs were 20.3pg/ml (P<0.001) and 12.9pg/ml (P<0.0001) respectively. Under the same conditions treatment with low and high doses of Vitamin D3 resulted in the downregulation of IFN-γ concentrations to 24.6pg/ml (P<0.001) and 11.9pg/ml (P<0.0001). When the PBMC was treated with half low and half high doses of M2000 and Vitamin D3, the concentration of IFN-γ was found to be 24.6pg/ml (P<0.001) and 11.9pg/ml (P<0.0001) respectively. P < 0.05 was considered statistically significant (Figure 2).

Effects of M2000, and Vitamin D3 on IL-6 expression

The baseline concentration of IL-6 in the PBMCs of the normal and positive controls were 3.2pg/ml and 15.7 pg/ml, respectively. After treatment and 24 hours of incubation of patients' PBMCs

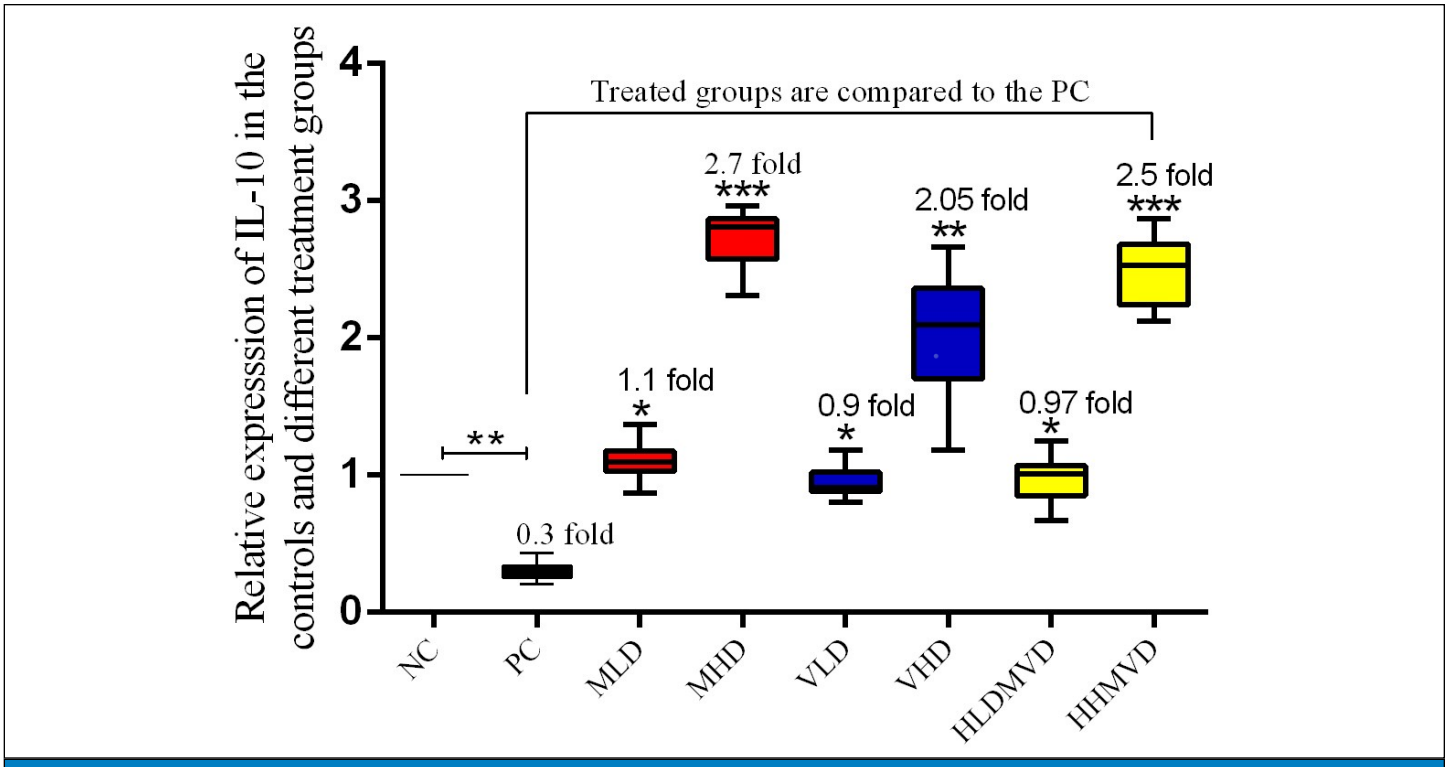

Figure 1: Relative expression of IL-10: Figure 1, shows the relative expressions of IL-10 in the normal control (NC), the positive control(PC),mannuronic acid low dose(MLD), Mannuronic acid highdose(MHD), Vitamin D3 low dose(VLD), Vitamin D3 High dose(VHD),Half low, Mannuronic+Vitamin-D3 (HLDMVD) and Half high Mannuronic+Vitamin D3 (HHDMVD). All the data are representative of three replicate Real-time PCR experiments.

with low and high doses of M2000, the levels of IL-6 cytokine concentration in the treated PBMCs were 12.2 pg/ml (0.001) and 8.0 pg/ml (0.0001), respectively. Under the same conditions treatment with low and high doses of Vitamin D3 resulted in the downregulation of IL-6 concentrations to 12.1 pg/ml (0.001) and 8.6 pg/ml (0.0001). When the PBMC was treated with half low and half high doses of M2000 and Vitamin D3, the concentration of IL-6 was found to be 13.7pg/ml($P<0.01$) and 7.1pg/ml ($P<0.0001$), respectively. $P < 0.05$ was considered statistically significant (Fig. 3).

Discussion

Gene and cytokine expression profile has been of great assistance in the understanding of IBD management and treatment. Many studies have implicated exaggerated cytokine and gene expressions as being responsible for Crohn's disease pathogenesis [21,22].

IL-10 is an anti-inflammatory cytokine that inhibits both antigen presentation and subsequent release of pro-inflammatory cytokines, thereby attenuating mucosal inflammation. It was observed in this research that the baseline expression of IL-10 in the positive controls was low compared with the normal control (Figure1), this finding was consistent with other previous research, where it was reported, that inflamed tissues and granulomas of CD show downregulation of IL-10 gene expression [23]. An inactivation of IL-10 in mice results in an increased production of IL-12 and IFN-γ. After treatment and incubation of the PBMCs with various doses of M2000, Vitamin D3 and a combination of the two drugs, there were significant upregulations in IL-10 gene expression compared with the positive controls. This was consistent with the IL-10 dose-response, placebo-controlled study, which reported that therapy with different doses of recombinant IL-10 (rHuIL-10) for seven days was beneficial in 46 refractory CD patients [24]. However, these preliminary results were not confirmed by placebo-controlled dose-response trials in patients with mild to moderately active or chronically active CD. The importance of

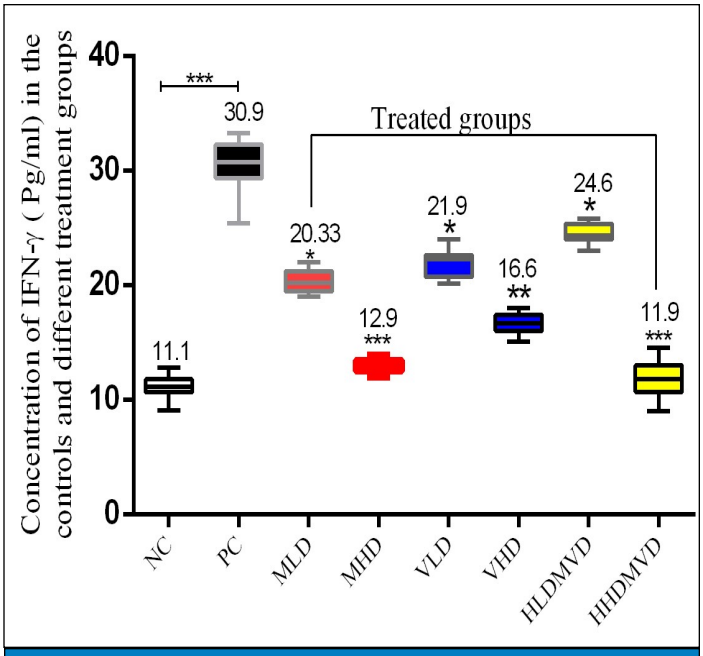

Figure 2: Concentration of IFN-γ(pg/ml): Figure 2,shows the concentrations of IFN-γ in the normal control (NC), the positive control(PC),mannuronic acid low dose(MLD), Mannuronic acid highdose(MHD), Vitamin D3 low dose(VLD), Vitamin D3 High dose(VHD),Half low, Mannuronic+Vitamin-D3 (HLDMVD) and Half high Mannuronic+Vitamin D3 (HHDMVD).All the data are representative of triplicate ELISA experiments.

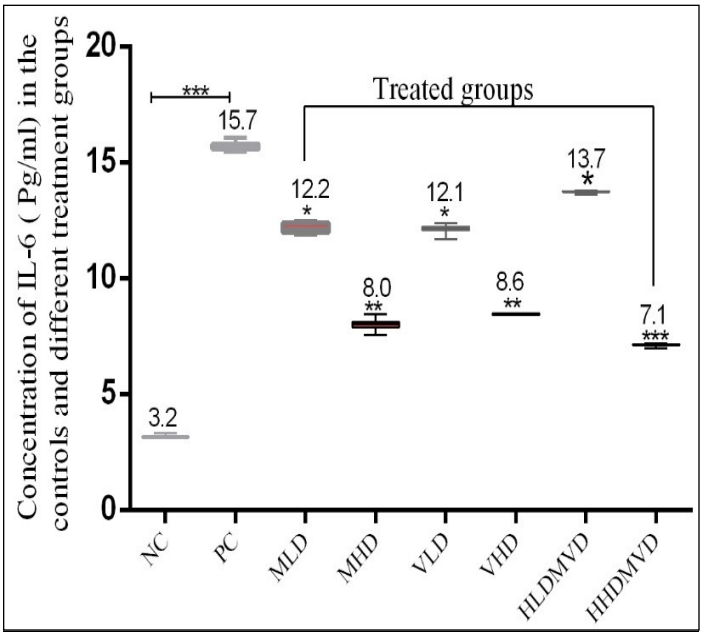

Figure 3: Concentration of IL-6(pg/ml): Figure 3,shows the concentrations of IL-6 in the normal control (NC), the positive control(PC),mannuronic acid low dose(MLD), Mannuronic acid highdose(MHD), Vitamin D3 low dose(VLD), Vitamin D3 High dose(VHD),Half low, Mannuronic+Vitamin-D3 (HLDMVD) and Half high Mannuronic+Vitamin D3 (HHDMVD).All the data are representative of triplicate ELISA experiments.

IL-10 production by B cells has been evidenced in IBD models and humans [11], Mizoguchi *et al.*, showed that B-regs can be responsible for the suppression and/or recovery from acquired immune-mediated inflammations by mechanisms that include IL-10 and TGF-β1 in IBD [10].

The IFN-γ is a key cytokine associated with Th1 T lymphocyte differentiation and various inflammatory responses. In our study, the baseline IFN-γ cytokine expression in the normal and positive controls was 11.1pg/ml and 30.7pg/ml, respectively (Figure 2). This increase is in agreement with the report of Papadakis et al, who stated that Crohn's disease shows a Th1 type of immune response with elevated pro-inflammatory cytokines, such as IL-12, TNF-α, and IFN-γ [25]. Our results have further shown that after 48 hours of treatment and incubation of patients' PBMC with M2000, Vitamin D3 and the combination of the two drugs, there was significant down-regulation in the concentration of IFN-γ between the treated PBMCs and positive controls (Fig.2). Reports of several *in vitro* studies have indicated that M2000 and Vitamin D3 can directly target CD4+ cells to promote Th2 development at the level of transcription, leading to a marked reduction in IFN-γ expression after the treatment [26]. Furthermore, *in vitro* studies of CD4+ T-cells of healthy controls and patients with Crohn's disease have shown that Vitamin D3 increases the production of anti-inflammatory cytokine IL-10 and decreases the production of pro-inflammatory IFN-γ, supporting a therapeutic role of Vitamin D3 in IBD, just as demonstrated in this study [27].

The IL-6 / STAT3 signalling pathways play an important role in CD pathogenesis. In this study, the baseline expression of IL-6 in the positive control was high compared to the negative control. This conforms with another research finding, which reported a high circulating level of IL-6 and sIL-6R in IBD patients and this correlates with disease activity [12]. The pathogenic role of the IL-6 and sIL-6R signalling in interfering with T-cell resistance to CD apoptosis was confirmed by blocking IL-6 trans-signalling. These research findings have shown that IL-6 can influence not only the chronic inflammatory pathways but also the relapses that arise in the pathology of CD [15]. The effects of IL-6 in IBD were demonstrated in Caco2 cells, where it was shown that IL-6 induces the activation of NF-kappa β and enhances the expression of intercellular adhesion 1-molecule. This adhesion molecule is im-

portant in the pathogenesis of IBD and is most likely required for the extra-intestinal manifestation in IBD [15]. Treatment of the PBMCs with various doses of Mannuronic acid, Vitamin D3 and the combination of the two drugs has significantly downregulated the concentration of IL-6 in the treated groups as compared to the PBMC of the positive controls (Figure 3). This downregulation in IL-6 expression observed in this study could be attributed to the blockage of the maturation pathways of APCs, especially, the dendritic cell by both M2000 and Vitamin D3 by constant inhibition of the actions of IL-12 and TNF-α on the antigen-presenting cells (APC). All the available research data have suggested that blockade of the IL-6 / STAT3 signalling pathways and the use of antibodies against IL-6R, as a new and promising therapeutic options in IBD treatment [15].

Conclusion/Recommendation

This research finding has shown the role of M2000 and Vitamin D3 as a novel non-steroidal anti-inflammatory, immunomodulatory and immunosuppressive drugs in the management of Crohn's diseases. This research results have shown that the optimal activities of M2000 and vitamin D3 were observed in the groups treated with high doses of the drugs. It's worthy to note that, the combination of half high doses of the drugs shown some level of immunosuppression, when compared to the combination half low doses of the drugs. It's therefore, recommended that a clinical trial be undertaken, to determine the effects of each of the regiments and their combination therapy in a clinical setting.

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I wish to express my sincere appreciation to the management of Tehran University of Medical Sciences, Management of TUMS-International Campus, for providing me with an enabling environment and the resources to carry out this research.

Conflict of interest

There is no conflict of interest to declare

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