Immunotherapeutic Effects of β-D Mannuronic Acid on IL-4, GATA3, IL-17 and RORC Gene Expression in the PBMC of Patients with Inflammatory Bowel Diseases

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ABSTRACT

Inflammatory bowel diseases (IBD) are chronic relapsing immune-mediated disorders that result from an aberrant immunological response. IBD comprises of Crohn's disease (CD) and ulcerative colitis (UC). The precise aetiology of IBD has not been fully understood, however, recent studies support the hypothesis that patients with IBD have a dysregulated immune response to endogenous bacteria in the gastrointestinal tract (GIT). The increasing number of hospitalisation coupled with the high economic burden faced by IBD patients, calls for more concerted research efforts, to design a potent and credible treatment option for these strata of patients.

This research was designed to test the efficacy and potency of β-D Mannuronic acid (M2000) in the treatment of IBD. Ten ml of blood was aseptically collected from 24 IBD patients and 24 normal controls. PBMC was isolated and stimulated with 1 μg/mL of LPS and incubated for 4 hours. The cells were later treated with 10 μg/mL or 50 μg/mL of Mannuronic acid and incubated for 24 hours at 37^0C under 5% CO2 and 100% humidity. After the incubation, RNA was extracted from the cells, cDNA was synthesised, and the expression of the gene was evaluated using quantitative real-time PCR.

The result indicated a significant down-regulation of RORC and IL-17 genes expression, while the expression of IL-4 and GATA3 genes were significantly up-regulated.

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These research findings have shown that M2000 a biocompatible agent, that has an immunotherapeutic, immunomodulatory and immunosuppressive effects on the PBMC of IBD patients.

Keywords: β-D Mannuronic acids; Crohn's disease; Immunosuppression; Immunotherapeutics; Ulcerative colitis

INTRODUCTION

Inflammatory bowel diseases (IBD) can be described as a group of idiopathic, chronic, and relapsing inflammatory disorders whose etiologic origins are yet to be understood, but many investigations have attributed the emergence of this disease to genetic susceptibility, immune dysregulations, environmental factors or bacterial infections.[1,2] IBD is of two subtypes, Ulcerative colitis (UC) and Crohn's disease (CD), each of these has its distinguishing characteristic. UC is a T-helper-2-like (Th2) inflammatory disorder, in which the morphological changes are restricted to the colon. In about 95% of patients diagnosed with UC, the rectum is involved.[3] CD is a T-helper-1 mediated (Th1) inflammatory disorder, which may extend from the oesophagus to the anus, but the ileocecal region and terminal ileum are the most commonly affected areas.[4] Inflammation in CD is usually transmural with a resultant fistula formation.[4,5]

The differentiation of naive T-cells plays a pivotal role in the development of IBD.[6,7] IL-4 is a major Th2 cytokine subset; its expression is controlled by the master regulator, the GATA3. The therapeutic intervention that upregulates the GATA3 gene expression will inadvertently affect the expression of IL-4.[4] RORC in human is the key transcription factor that orchestrates the differentiation of Th17 effector cell lineage. RORC induces transcription of the genes encoding for IL-17 and the related cytokine IL-17F in naive CD4+ T helper cells and is required for their expression in response to IL-6 and TGF-β, the cytokines known to induce IL-17.[8] Mice with RORγt deficient T cells have attenuated autoimmune disease and lack tissue-infiltrating Th17 cells. Altogether, these findings suggest that RORγt or RORC are the key regulator of immune homoeostasis and highlight their potential as a therapeutic target in IBD.[8]

It is on record that most of the available immune modifiers, as well as anti-inflammatory drugs used in the management of IBDs, have some inadequacies and side effects, such as over-dependence and damage to some vital organs of the body, such as liver, kidney, lungs, and GIT, thereby exposing the patients to the risk of developing colorectal cancer and other debilitating diseases.[9-11] Therefore there is an urgent need, to develop safe, effective, and affordable therapeutic options that could replace the existing drug regimens.

β-D mannuronic acid, which is also referred to as M2000, is derived from sodium alginate, which comprised of D-mannuronate (M block) and L-guluronate (G-block) linked by 1→4 glycosidic linkage. They are synthesized from a naturally occurring brown sea alga. It is a biocompatible material that has wider medical applications and has successfully been used for the past four decades without any reported cases of adverse effects.[12] β-D mannuronic acid is an important copolymer of sodium alginate and has been patented with the number (DEU: 102016113018.4). The drug has so far demonstrated its enormous immunomodulatory and immunotherapeutic potentials in diseases,[13] such as multiple sclerosis (MS), nephrotic syndrome (NS), rheumatoid arthritis (RA) and immune complex glomerulonephritis.[14-18] Mirshafiey et al, have shown in their works that β-D Mannuronic acid (M2000) can exhibit its action through constant inhibition of the activity of matrix metalloproteinase-2, prevention of immune cell infiltrations and down-regulation of IL-6 and Immunoglobulin production.[19] β-D Mannuronic acid has been reported to have encouraging results in other experimental models of autoimmune diseases.[20] The aim of this research was to identify a novel immunosuppressive and anti-inflammatory agent, that can serve as a therapeutics option in the treatment of IBD. This research was designed to assess the therapeutic efficacy of M2000 on IL-4, GATA3, IL-17 and RORC gene expression in the PBMC of IBD patients.

MATERIALS AND METHODS

Study Population

The research was conducted on 48 subjects which include 24 newly diagnosed patients with moderate/mild IBD and 24 normal controls. The participants were recruited from the Digestive Disease Research Institute (DDRI), Shariati Hospital and Gastroenterology Department, Firosgar Hospital, Iran University of Medical Sciences, Tehran, from February 2016 to April 2017. The mean age ranges were 31.0417 ± 5.39306 for the patients and 29.8333 ± 5.70024 for the normal controls. (Table 1)

Inclusion Criteria

All the research participants were recruited based on the following inclusion criteria.
1. Persistent stomach upsets and pain
2. Watery and bloody stool
3. Presence of microcytic anaemia
4. Increased thrombocytosis
5. Increased leukocytosis
6. Increased C-Reactive Protein
7. Increased Erythrocyte Sedimentation Rate
8. Positive endoscopic and colonoscopic evaluations.
9. Patients should not be on any immuno-suppressive and anti inflammatory agents.

Exclusion Criteria

The list of the exclusive criteria for the research are as follows:
1. Patients diagnosed with any form of infectious diseases
2. Pregnant and lactating mothers
3. HIV positive patients
4. Drugs users
5. Smokers
6. Patients already on immunosuppressive, anti-inflammatory regimens and steroids.

Ethical Approval and Informed Consent

The research study was approved by the ethical committee of Tehran University of Medical Sciences (TUMS) Tehran, under the approval number, IR.TUMS.REC.1394.2075. Written informed consent was obtained from all the research participants and the research was carefully conducted in line with the international best practice in conformity with the international standard ethical protocol.

Preparation of M2000

M2000 (β-D Mannuronic acid) is a small molecule, which has a molecular formula of $C_6H_{10}O_7$ and a molecular weight of 194.139 Da. It was prepared from sodium alginate (Sigma-Aldrich, St Louis, MO, USA) with reference to the method of Fattahi et al.[20] The purity of M2000 was subsequently validated by the characterization of the hydrolytic products, using Fourier Transform Infrared (FTIR) spectroscopy and carbon-13 nuclear magnetic resonance (13C-NMR) spectroscopy.

Blood Collection and PBMC Isolation

Ten ml of blood sample was obtained from 24 IBD patients and 24 healthy controls. Using Ficoll-Paque centrifugation method, (Amersham Pharmacia Biotech, Uppsala-Sweden), the PBMC were isolated within 2-3 hours of the blood collection from the research participants. The isolated PBMCs were suspended in RPMI 1640 (GIBCO) with 10% FBS (GIBCO) and 1% pen/strep. The cells were counted using improved Neubauer hemacytometer counting chamber.

Experimental Groups

The experimental groups consisted of four groups, the normal controls (NC), the positive controls (PC), the Mannuronic acid low dose (MLD) and the Mannuronic acid high dose (MHD). The MLD groups were treated with 10 μg/mL, while the MHD groups were treated with 50 μg/mL.

Cell Culture Stimulation and Treatment of PBMC by LPS and M2000

In a 24-well culture plate, 1.5×10^6 cells were added to each well. The cells were stimulated with 1 μg/mL of LPS (Sigma –Aldrich, Germany) and incubated for 4 hours at $37^{\circ}C$, in 5% CO2 and 100% humidified air. After 4 hours of incubation, the PBMCs were treated with 10 μg/mL and 50 μg/mL of low and high doses of Mannuronic acid respectively. The cells were incubated for an additional 24 hours at $37^{\circ}C$, in 5% CO2 and in 100% humidified air.

RNA Extraction and cDNA Synthesis

The cells were harvested from the cell culture plates into 2ml Eppendorf tube and centrifuged at 12,000

Table 1. Demographic and clinical characteristic of participants in the study on the effects of β-D Mannuronic acid on gene expression in peripheral blood mononuclear cells (PBMC) of patients with inflammatory bowel diseases (IBD)

Topics	NC (n=24)	PC (n=24)	*p*-value
Age (Mean ±SD)	29.8333±5.70024	31.0417± 5.39306	0.771
Sex (Male/female)			0.422
	10/14	14/10	
Duration of Disease (Months)	-	19.7917± 6.54070	-
Disease Serotype	-	10 (41.7%) / 14 (58.3%)	-
CD/ UC			
Drugs regimens	-	6	-
Microcytic anaemia	-	21/24 (87.5 %)	-
Increased thrombocytosis	-	23/24 (95.33%)	-
Increase Leukocytosis	-	22/24 (91.66%)	-
Increase CRP	-	23/24 (95.33%)	-
ESR	-	22/24 (91.66%)	-
Colonoscopy or endoscopy/biopsy	-	24/0(100%)	-
MRI/CT SCAN	-	5/24 (20.83%)	-

RCF for 10 minutes to separate the cells from the supernatant. The supernatants were stored at -70^{0}C until ready for cytokine assay. Total RNA was extracted from 2×10^6–3×10^6 cells, using GeneAll Hybrid-RTM kits, Cat.No.305-101 (Songpa-GU, Seoul, Korea 138-859), based on the manufacturer's instructions. The final concentration and purity of the total RNA were measured by NanoDrop 1000 UV–Spectrophotometer (Thermo-Fisher Scientific, Waltham, MA, USA). The total RNA was stored at -70^{0}C prior to cDNA synthesis.

The cDNA synthesis was performed using 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 integrity of the synthesized cDNA was evaluated by gel electrophoresis of PCR products. (Figure 1) The cDNA was stored at -20$^°$C until required for quantitative real-time PCR.

Quantitative Real-Time PCR

The Quantitative real-time PCR was performed using SYBR$^®$ Premix Ex Taq™ II (Takara Co., Ltd.), with specific primers (Sigma-Aldrich; Table 2) based on the provided guideline. The IL-4, GATA3, IL-17, RORC and GAPDH gene expression was performed using StepOnePlus™ Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA). The relative expressions of target gene mRNA, compared to the endogenous control, (GAPDH mRNA) were evaluated using the ΔCT method, with reference to each amplification plot (fluorescence signal vs cycle number). The mean difference (ΔCT) between the

Table 2. Primer Sequences for the effects of β-D Mannuronic Acid on IL-4, GATA3, IL-17 and RORC gene expression in the peripheral blood mononuclear cells (PBMC) of patients with inflammatory bowel diseases (IBD)

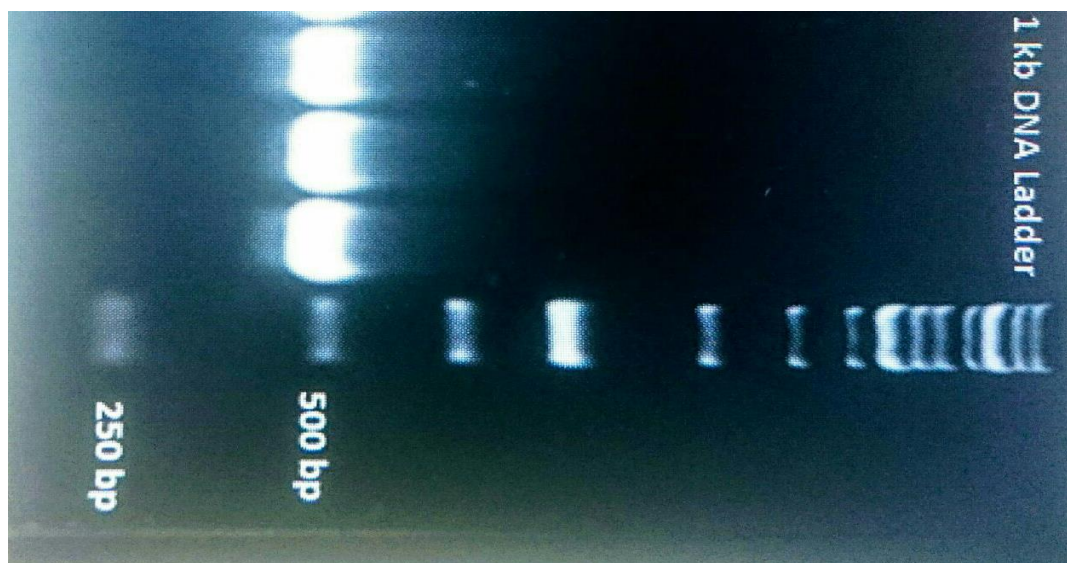| Primer	Forward Sequence	Reverse Sequence
IL -4	CCAACTGCTTCCCCCTCTG	TCTGTTACGGTCAACTCGGTG
GATA3	GAGGGCTGGTTTCCTTGACTG	AAAAAGGGGCGACGACTCTG
IL-17	CTGTCCCCATCCAGCAAGAG	AGGCCACATGGTGGACAATC
RORC	GTGGGGACAAGTCGTCTGG	AGTGCTGGCATCGGTTTCG
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

Figure 1. Gel electrophoresis of PCR Products on the effects of β-D Mannuronic Acid on IL-4, GATA3, IL-17 and RORC Gene Expression in the PBMC of Patients with Inflammatory Bowel Diseases. This was performed using 1% agarose gel, 0.5x TAE Buffer, ExcelBand of 1 KB (0.25-10 KB) DNA ladder, 10x loading buffer and Redgel. The PCR products show a band of 500 base pairs (bp)

values in the replicate samples of target genes and that of the endogenous control (GAPDH mRNA), was calculated. The changes in the expressions of the target genes and the normal controls were calculated using, $\Delta CT_{Patients}-\Delta CT_{Controls}=\Delta\Delta CT$. This was further expressed as a relative fold change or expression of the gene in the patients compared to the normal controls. ie, $2^{-\Delta\Delta CT}$.

Statistical Analysis

The statistical analysis was carried out by SPSS software (22.0; IBM Corporation, Chicago, IL, USA). All the data were expressed as the mean, standard deviation and $p<0.05$ was statistically considered as significant. The Kolmogorov-Smirnov test was used to check the normality of all the data. The analysis of variance (ANOVA) test was used to compare the quantitative variables between the groups and the Post hoc turkey was used to determine significant differences in the gene expression level between the positive controls and treated groups. GraphPad Prism software version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA), was used to design all the graphs.

RESULTS

The results of this study as presented in Figure 2-5 has demonstrated the considerable potentials of β-D Mannuronic acid (M2000) in immunomodulation and regulations of most inflammatory mediators that are involved in the pathogenesis of various inflammatory processes, most especially in IBD.

Effect of M2000 on IL-4 Gene Expression

Figure 2 shows IL-4 gene expression in the normal control, positive control and treated patients' PBMCs. A fold change of 0.2 ($p<0.0001$) in IL-4 expression in the PBMCs of the positive control was observed when compared to the normal control. After treatment and 24 hours of incubation with low and high doses of M2000, the level of IL-4 gene expression has been up-regulated to 1.6 ($p<0.001$) and 2.6 (0.0001) fold change, compared to the positive control.

Effect of M2000 on GATA3 Gene Expression

The level of GATA3 gene expression in the positive control was very low when compared to the normal control.

The relative gene expression of GATA3 was 0.15 ($p<0.0001$) fold change in the positive controls' PBMC, compared to the normal control. After 24 hours of treatment and incubation with various doses of M2000, there was significant upregulation of 1.5 ($p<0.001$) and 2.5 ($p<0.0001$) fold changes in GATA3 gene expression, when compared to the positive control (Figure 3).

Effect of M2000 on IL-17 Gene Expression

The level of IL-17 gene expression in the positive controls' PBMC has indicated a fold change of 4.1 ($p<0.0001$) when compared to the normal control. After treatment with various doses of M2000, and 24 hours of incubation of the patients' PBMC, the levels of IL-17 expressions were down-regulated to 2.8 ($p<0.001$) and 1.4 ($p<0.0001$) folds respectively (Figure 4).
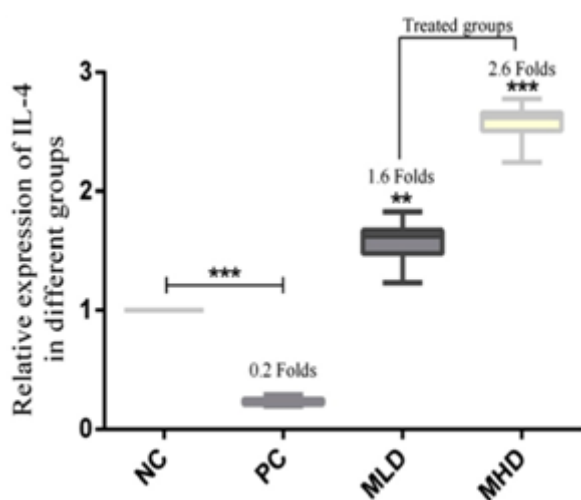
Effect of M2000 on RORC Gene Expression

The level of RORC gene expression in the PBMC of the positive control was 5.0 ($p<0.0001$) fold change when compared to the normal control. After treatment with various doses of M2000, and incubation for 24 hours, the fold expression of 4.1 ($p<0.001$) and 2.0 ($p<0.0001$) were observed in the relative expression of RORC gene. These are significant down-regulations in relative expressions between the treated and positive control. (Figure 5).
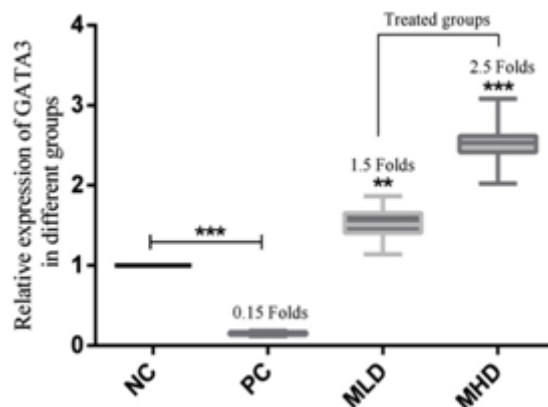

Figure 2. The effects of β-D mannuronic acid on IL-4 gene expression in peripheral blood mononuclear cells (PBMC) of patients with inflammatory bowel diseases (IBD) in different groups: The relative expression of IL-4 in the PBMC of the positive control (PC) was 0.2 fold when compared to the normal control (NC). After treatment with 10μg/ml and 50μg/ml of M2000 and incubation for 24 hours, the relative expression of IL-4 has indicated a 1.6 ($p<0.001$) and 2.6 (0.0001) fold change in the treated groups compared with the positive control. Statistically, $p<0.05$ is considered significant (NC=Normal control, PC=Positive control, MLD=Mannuronic acid low dose, MHD=Mannuronic acid high dose, ** $p<0.001$,* $p<0.0001$). All the data are representative of three replicates QRT-PCR experiments**

Figure 3. The effects of β-D Mannuronic acid on GATA3 gene expression in peripheral blood mononuclear cells (PBMC) of patients with inflammatory bowel diseases (IBD) in different groups: The relative expression of GATA3 in the PBMC' of the positive control was 0.15 (0.0001) folds when compared to the normal control. After treatments of the patients' PBMCs with 10μg/ml and 50μg/ml of M2000 and incubation for 24 hours, the relative expression of GATA3 has indicated fold change of 1.5 ($p<0.001$) and 2.5 (0.0001) in the treated groups compared to the positive control. $p<0.05$ is statistically considered significant. (NC =Normal control, PC=Positive control, MLD=Mannuronic acid low dose, MHD=Mannuronic acid high dose, ** $p<0.001$, *** $p<0.0001$). All the data are representative of three replicates QRT-PCR experiments.
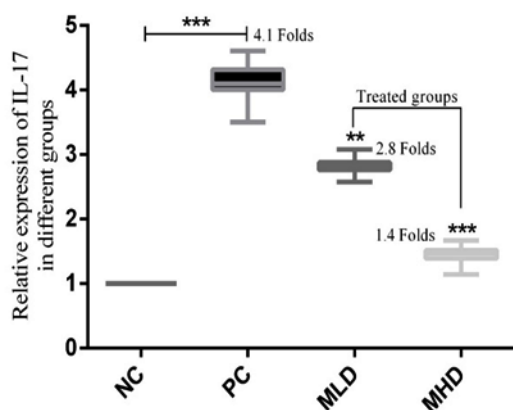
Figure 4. The effects of β-D mannuronic acid on IL-17 gene expression in peripheral blood mononuclear cells (PBMC) of patients with inflammatory bowel diseases (IBD) in different groups: The relative expression of IL-17 in the PBMC of the positive control was 4.1 fold, relative to the normal control. After the treatment with 10μg/ml and 50μg/ml and 24 hours of incubation, the fold expressions in IL-17 were 2.8 ($p<0.001$) and 1.4 ($p<0.0001$) folds, in the treated groups compared to the positive control. P<0.05 is statistically considered significant. (NC= Normal control, PC = Positive control, MLD =Mannuronic acid low dose, MHD = Mannuronic acid high dose, ** $p<0.001$, *** $p<0.0001$). All the data are representative of three replicates QRT-PCR.

Figure 5. The effects of β-D mannuronic acid on RORC Gene expression in peripheral blood mononuclear cells (PBMC) of patients with inflammatory bowel diseases (IBD) in different groups: The relative expression of RORC in the positive control (PC) was 5.0 fold expression when compared to the normal control (NC). After the treatment of the patients' PBMCs with 10 μg/ml and 50μg/ml of M2000 and incubation for 24 hours, the relative expression of RORC was down-regulated to 4.1 ($p<0.001$) and 2.0 ($p<0.0001$) folds change respectively. $p<0.05$ |is statistically considered significant. (NC=Normal control, PC=Positive control, MLD=Mannuronic acid low dose,MHD=Mannuronic acid high dose, ** $p<0.001$, *** $p<0.0001$). All data are representative of three replicates QRT- PCR.

DISCUSSION

New insights into the pathogenesis of IBD which focus on gene expression profile, has achieved tremendous success in the understanding of IBD pathogenesis. Many studies have demonstrated overexpression of transcription factors and pro-inflammatory cytokines as the major causes of IBD.[21,22] In our present study, there was a significant downregulation of IL-4 gene expression in the positive control in contrast to the normal control (Figure 2). This result is in agreement with work of Karltunnen R, et.al, where it was reported that the levels of IL-4 mRNA are mainly downregulated in active IBD.[23] Treatment of the patients' PBMCs with different doses of M2000 was accompanied with proportional up-regulation in the level of IL-4 gene expression (Figure 2). It is worthy to note that, IL-4, as an anti-inflammatory cytokine, possesses immunoregulatory and immunosuppressive effects in the gut, through
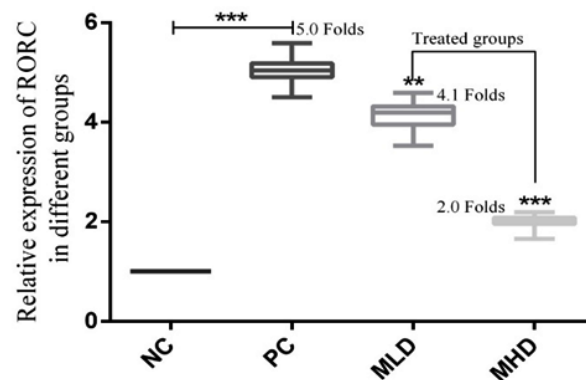
mediating the differentiation of naive T cells to the Th2 subset. Reductions in the levels of IL-4 and IL-4 mRNA, which is mainly found in IBD may be associated with defective immunosuppression and anti-inflammatory mechanisms that may contribute to the pathogenic cascade leading to inflammation.[24] IL-4 seems to be decreased more in Crohn's disease (CD) patients than in ulcerative colitis (UC) patients, indicating again different immunopathogenic mechanisms in the two diseases.[24,25]

The GATA3 relative expression in the positive control was found to be low when compared to the normal control in our study (Figure 3). This low expression might be due to the inhibitory effects of both RORC and Tbet, the master regulators of Th17 and IFN-γ, on GATA3 gene expression, resulting in the down-regulation of IL-4 cytokine expression.[26] This result is in conformity with other research findings, where low GATA3 gene expression was recorded, especially in UC. Th2 differentiation requires the

GATA3 master regulator and the deficiency of GATA3 expression leads to loss of Th2 cell expression, resulting in low expression of IL-4.[26] Human heterozygous for GATA3, such as in IBD have decreased frequencies of Th2 cells and other anti-inflammatory cytokines.[27] Intervention with M2000 could serve as a therapeutic option in the treatment of the patient with the deficiency or low expression of GATA3 gene. As demonstrated in our study, there was a significant up-regulation in the level of GATA3 gene expression, after 24 hours of incubation with M2000 (Figure 3). This is an interesting development, because increases in the GATA3 gene expression profile, will inhibit the Th1/Th17 differentiation pathway by obstructing the normal functions of RORγt/Tbet, thereby blocking the exaggerated productions of IFN-γ, IL-17, TNF-α and other inflammatory mediators responsible for IBD development.[28]

IL-17 is produced mainly by Th17 cells, although CD8$^+$ T cells are also able to produce this cytokine during chronic inflammation.[29] IL-17 acts as a key mediator in delayed-type immune reactions by increasing chemokine production and recruiting monocytes and neutrophils to the site of inflammation.[30] In our study, there was a significant up-regulation in the level of IL-17 gene expression in the PBMC of IBD patients compared to control. This result was in conformity with other reported research findings in human, where elevated levels of IL-17 in active CD and UC were observed.[31] Most recently, it has been shown that in CD patients, increased numbers of circulating IL-17 and IFN-γ-producing CD161$^+$ memory cells were present, and these cells constitute a high percentage of colonic mucosal cells.[32] The effects of M2000 in IL-17 down-regulation is paramount in the treatment of IBD. In our study after 24 hours of treatment and incubation with low and high doses of M2000, there was a significant down-regulation of IL-17 gene expression when compared to the positive control. M2000 was able to downregulate IL-17 gene expression, probably by antagonizing and selective blockade of Toll-like receptor (TLR2 and TLR4) signaling to naive CD4$^+$T-cells, based on inhibition of its differentiation and down-regulation of Th1/Th17 proliferation.[33]

RORγt or RORC in human is the master regulator of the Th17 differentiation. Individual deficient in RORC has limited Th17 differentiation, while over-expression RORC/RORγt induced IL-17 expression in the absence Th17 polarising cytokines.[8] In our study, there was a five-fold increase in the relative expression of RORC in positive control when compared to the normal control (Figure 5). The relative over-expression of RORC observed in this study may also be responsible for high expression of IL-17 observed in this study as well. This may be due to the synergistic action between RORC and IL-17, in increasing the expression of IL-17, while inhibiting both the expression of Th1, IFN-γ Tregs and FOXP3 gene expression.[34] It was observed in our study that after 24 hours treatment and incubation with β-D Mannuronic acid (M2000), there was a significant down-regulation of the RORC gene expression compared to the positive control. This down-regulation in the RORC expression as reported in other studies could result in up-regulation of FOXP3 expression with the resultant iTreg up-regulation,[35] and this is pertinent and important in treatment and overall management of IBD.

To the best of our knowledge, this is the first research that assessed the effect of β-D Mannuronic acid (M2000) on the gene expression of IL4, GATA3, IL-17 and RORC in patients with IBD, and the result of this research has further shed more light on the role of M2000 as a novel immunosuppressive, immunomodulatory and non-steroidal anti-inflammatory drug in the management of IBD. M2000 was able to significantly reduce the relative expression of IL-17 and RORC gene expression while up-regulating the relative gene expressions of IL-4 and GATA3 in the PBMC of IBD. We wish to conclude by highlighting that our research is an *ex vivo* study as such, we recommend that a well-tailored multi-centred *in vivo* research study should be undertaken to determine the effects of M2000 in the overall management and treatment of IBD patients under *in vivo* conditions.

ACKNOWLEDGEMENTS

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