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Immunomodulatory Effects of Vitamin D3 on the Expression of IL-6, IFN-γ and T-BET in Inflammatory Bowel Diseases

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Abstract

Inflammatory bowel diseases (IBD) is an immune mediated dysfunction in a genetically susceptible host. Crohn's diseases (CD) and ulcerative colitis (UC) are serotypes of IBD. They are important cause of gastrointestinal diseases. Patients with IBD are predisposed to colon cancer. It is therefore not only imperative that patients with IBD have regular colon cancer screenings and monitoring of inflammatory markers, but efforts should be doubled in designing potent treatment options for IBD management. This research aimed to assess the immunomodulatory effects of Vitamin D3 (1,25(OH)2D3) on the expressions of T-bet, IL-6 and IFN-γ in IBD patients. Fifteen (15) ml of whole blood was aseptically collected from 20 IBD patients and 20 healthy controls. Peripheral blood mononuclear cells were isolated and stimulated with 1µg/ml of LPS in a cell culture plate and incubated for 4 hours. The cells were later treated with 10^{-10} and 10^{-8} M of $1\alpha,25(OH)^2$ D3 and incubated at 37^0C, under 5% CO2 and 100% humidity. The RNA extractions, cDNA synthesis, ELISA, primer design and qRT-PCR was later carried out. The result findings indicated a significant down-regulations of IL-6, IFN-γ and T-bet, gene expressions. The research has shown that 1,25(OH)2D3 do not just possess immunomodulatory activities but also possess immunoregulatory and anti-inflammatory effects and thus, it may be used in the management of IBD patient.

Keywords: 1,25(OH)2D3, IBD, Ulcerative colitis, Crohn's disease, immunomodulation

Introduction

Inflammatory bowel disease (IBD) is characterised by severe inflammation of the small bowel and/or the colon leading to recurrent diarrhoea and abdominal pain. IBD has two different subtypes, Crohn's disease (CD) and ulcerative colitis (UC). Despite both being chronic and relapsing inflammatory diseases of the bowel, they can be differentiated by the site of the inflammation in the gastrointestinal tract (GIT) and by the nature of the histological alteration in the intestinal wall (Ogunbi et al.,9981). Anatomically, CD can affect the entire GIT from mouth to anus, although it commonly affects the terminal ileum and colon. UC is restricted to the rectum, colon and caecum. Microscopically, CD is transmural and often discontinuous, while UC affects only the intestinal mucosa in a continuous pattern (Ogunbi *et al.,* 9981). Cytokines are key signals in the intestinal immune system and are known to participate in the disruption of the so-called normal state of controlled inflammation (physiological inflammation of the gut) (Rogers *et al.,*1971). Cytokines are small peptide proteins produced mainly by immune cells that facilitate communication between cells, stimulate the proliferation of antigen specific effector cells, and mediate the local and systemic inflammation in an autocrine, paracrine, and endocrine pathways (Mir-Madjlessi *et al.,* 1985). In IBD, the innate immune response plays critical roles, activated dendritic cells (DC) and macrophages secrete several cytokines that actively regulate the inflammatory response in UC and CD. Once secreted by these antigen presenting cells (APC), these cytokines triggers the differentiation of

many T cells subset, resulting in the activation of the adaptive immune response (Mir-Madjlessi *et al.,* 1985). IBD has been characterized by a T cell dysregulation where clearance of over reactive and autoreactive cells are disturbed, in addition to an imbalance of Treg/Th1, Th2 and newly described Th17 cells population in the activated state. The lack of appropriate regulation from T cells, or an over-production of effector T cells, participates in the development and exacerbation of IBD pathogenesis (Hendrickson *et al.,* 2002).

Altogether, APCs, Th1, Th2, T regulatory cells and most recently characterized Th17 and their cytokine products play a complex role in IBD (Hendrickson *et al.,* 2002). These cellular interactions are modulated by both traditionally studied cytokines (such as TNF-α, IFN-γ, IL-1, IL-6, IL-4, IL-5, IL10, TGF-β) and others recently characterized (like IL-13, IL-12, IL-18, IL-23), considered to be either pro or anti-inflammatory, depending on their roles in the inflammatory cascade (Sewell *et al.,* 2010). Most important inflammatory responses in IBD or other inflammatory disorders are mediated by cytokines, such as the regulation and the production of inflammatory mediators, reactive oxygen metabolites, nitric oxide, leukotrienes, platelet-activating factor, and prostaglandins, activation of the nuclear factor κB (NF-κB) and inhibition of apoptosis (Sewell *et al.,* 2010). CD is a Th1 T cell mediated inflammatory disorders, which is characterized by enhanced production of IFN-γ and TNF-α. IL-12 and IL-23 govern the Th1 differentiation which in combination with IL-15, IL-18 and IL-21 induced the stabilization of polarized Th1. On the other hand, UC is a Th2-like inflammatory condition, characterized by the less polarized local immune response, but it is characterized by CD1 reactive natural killer T cell production of IL-13 and Th2 cytokine production. The aim of this research is to access the immunomodulatory effects of 1α,25(OH)2D3 (Vitamin D3) on IL-6, IFN-γ, FOXP3, and T-Bet gene in the PBMC of IBD patients.

Materials and Methods

Study type:

The research was Laboratory and hospital-based ex vivo investigations. Demographic information was obtained from research participants by means of questionnaire for patients in Digestive Diseases Research Institute (DDRI) Shariat Hospital, Tehran.

Research Participants

These include patients diagnosed with moderate/mild IBD and normal apparently healthy individuals served as controls. The research was conducted on 48 participants, these includes: 24 subjects diagnosed with mild/moderate IBD, consisting of 14 (58.33%) males and 10 (41.66%) females and 24 normal healthy controls, consisting of 10 (41.66%) males and 14 (58.33%) females. The mean age ranges were 31.0417± 5.39306 for the patients and 29.8333±5.70024 for the normal controls.

Sample Collection and PBMC Isolation

Ten (10) ml of blood sample were aseptically collected from each of the research participants with the aid of a venject. The PBMC isolation was performed using Ficol-Paque centrifugation method. The blood sample was emptied into 50 ml Falcun tube and diluted in an equal volume of PBS. Three (3) ml of Ficol medium was added into a fresh 15 ml Falcun tube. Ten (10) ml of diluted blood was layered on the Ficol medium with the aid of a sterile Pasteur pipette. The blood was allowed to stand for 1 to 2 minutes and then centrifuged at 2500rpm or 600g for 20 minutes at room temperature without a break.

The blood separated into parts, an upper part containing the plasma, an interphase containing the PBMC, and the lower part, containing the red cells. The PBMC was carefully aspirated into a new sterile 15 ml Falcun tube. PBS was added into the isolated PBMC to the 14 ml volume mark and then centrifuged at 1300rpm for 7 minutes. This was repeated one more time. The supernatant was discarded and 3 ml of complete culture medium containing RPMI 1640 (GIBCO) with 10% FBS (GIBCO) and 1% pen/strep was added to the PBMC. Using an improved Neubauer hemacytometer counting chamber the cells were counted.

Cell Culture: Cells Stimulation and Treatment

In a 24-well culture plate, 1.5 x10^6 PBMCs were seeded in each well. The cells were stimulated with 1μg/ml of LPS and incubated for 4 hours at 37^{0}C, 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 Mannuronic acid, 10^{-10}M and 10^{-8}M of low and high doses of Vitamin D3 and Half low doses of Mannuronic acid plus Vitamin D3 (5µg/ml + 5 X10^{-11}M) and Half high doses of Mannuronic acid plus Vitamin D3 (25µg/ml + 5x10^{-9}M) respectively, and Then incubated for an additional 24 hours at the same culture conditions.

RNA Extraction

The PBMCs were harvested from the cell culture plates into 2ml Eppendorf tube, The cells were centrifuge at 12,000 RCF for 10 minutes to separate the cells from the supernatants. The supernatants were stored at -70^{0}C until ready for cytokines assay. Total RNA was extracted from 2×10^{6}– 3×10^{6} cells, using GeneAll® Hybrid-R™ kits Cat. No. 305-101 (Songpa-Gu, Seoul, Korea 138-859).

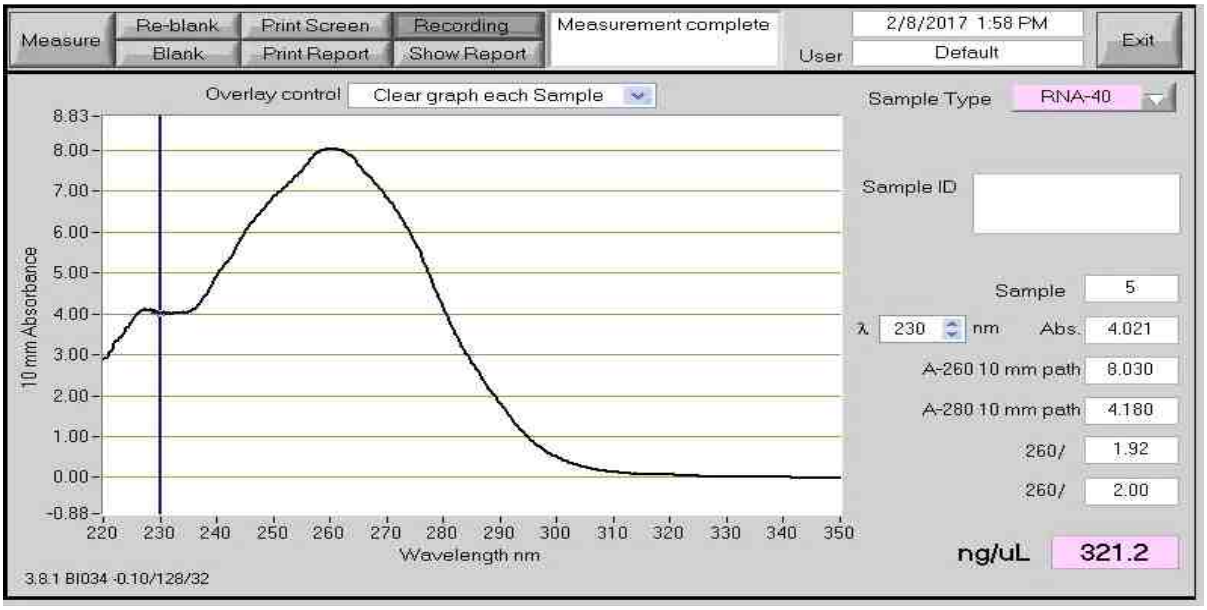

Figure 1a: Nanodrop result for Normal Control sample (NC): RNA concentration is 321ng/µl, 260/280 is 1.92, 260/230 is 2.0

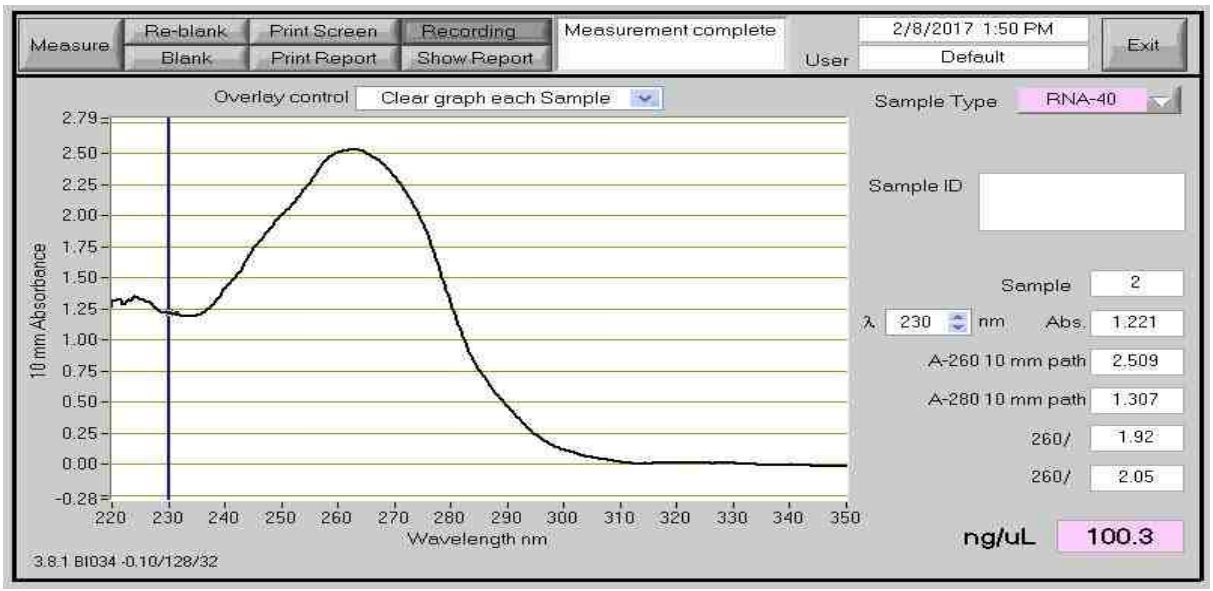

Figure 2a: Nanodrop for Ulcerative colitis patient sample (PC): RNA concentration is 100 ng/µl. 260/280 is 1.92, 260/230 is 2.05.

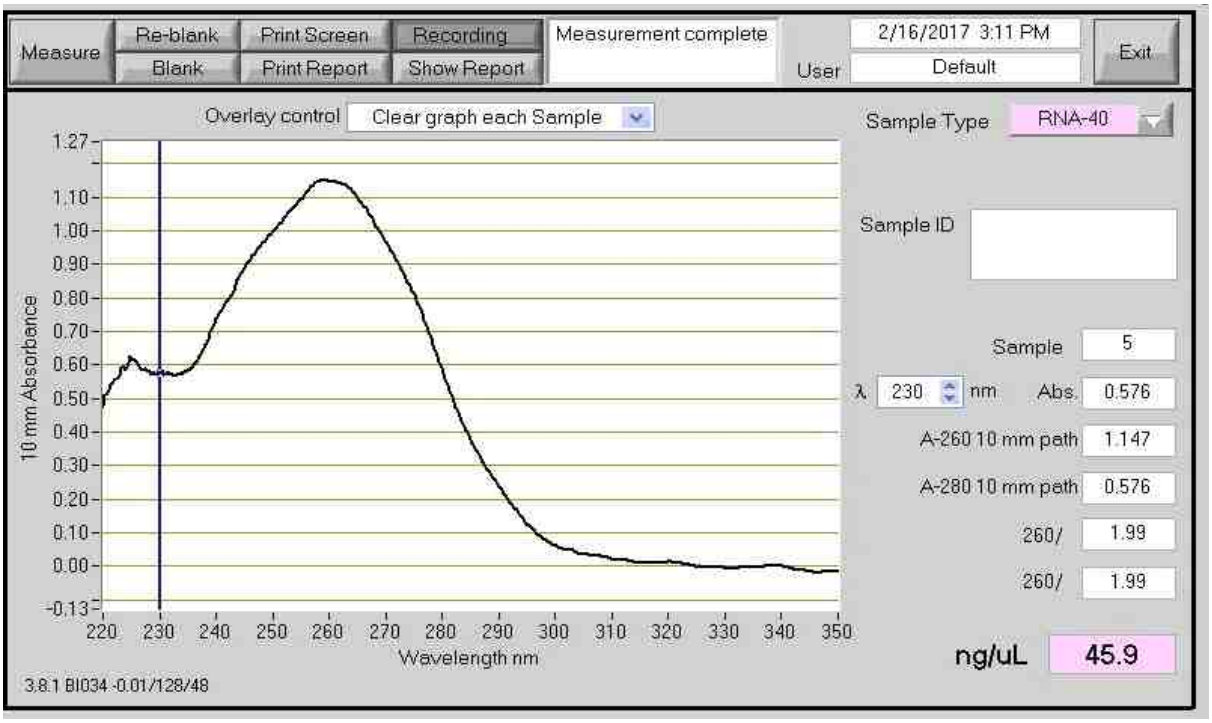

Figure 3*a*: Nanodrop for Crohn's disease patients' sample (PC): RNA concentration is 45.9 ng/µl, 260/280 is 1.99, 260/230 is 1.99

cDNA synthesis

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

Determination of IFN-γ and IL-6 Using ELISA Technique

The concentration IFN-γ and IL-6 in the supernatants from 24-hours PBMCs cell cultures were determined. Using specific Human IFN-γ ELISA Ready-Set-Go kit^{R}, catalogue number: 88-7316 and Specific Human IL-6 ELISA Ready-set-Go kit^{R}, catalogue number: 88-7066 (EBioscience, Inc, an Affymetrix, California USA). This evaluation was carefully carried out in line with the manufacturer's instructions.

Real-time PCR for Determination of T-Bet Expression

The Quantitative real-time PCR was performed using SYBR® Premix Ex Taq™ II (Takara Co., Ltd.) with all the specific primers (Sigma-Aldrich), based on the provided guidelines. The gene transcription analysis of T-bet, and GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) as the housekeeping gene, were performed using StepOnePlus™ RT-PCR Systems (Applied Biosystems, Foster City, CA, USA). The relative expressions of target gene mRNA compared against the endogenous gene, GAPDH mRNA, were measured using a ΔCT method with reference to each amplification plot (fluorescence signal vs cycle number). The mean difference (ΔCT) between the values of replicate samples 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 expressed as relative fold change in the patients compared to the normal and healthy control. ($2^{\wedge-\Delta\Delta CT}$)

Results

The relative expression of T-bet in different group

The baseline folds expression of T-bet in positive controls was 3.05 fold. After 24 hours of treatment with low and high doses of Vitamin D3 treatment, the relative expression were 2.24 ($P<0.01$) and 1.63 ($P<0.001$),

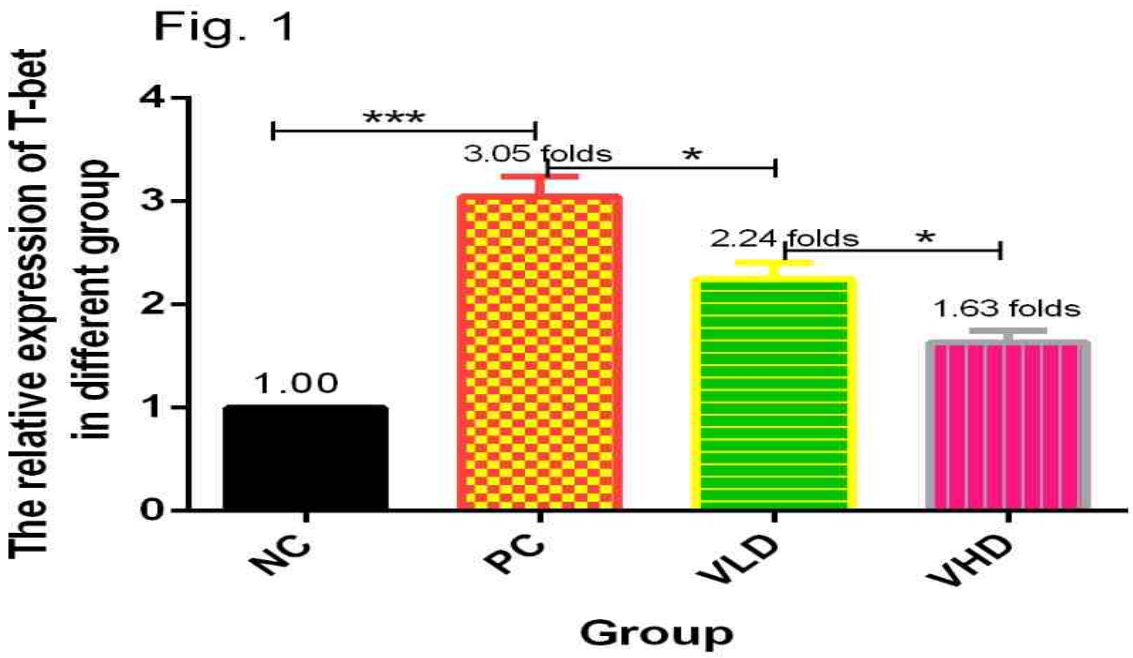

Figure 1b: Shows the T-bet relative gene expression in both the controls and the treatment group. **Note**: the PC is compared with the NC, and all the treatment groups are compared with the PC). P-value < 0.05 was considered statistically significant. (NC = Normal control, PC = Positive control, VLD=Vitamin D3 low dose. VHD= Vitamin D3 high dose * P<0.01, **P<0.001, ***P<0.0001). All the data are representative of three replicate qRT-PCR experiments.

The IFN-γ Expression in Different Groups

The IFN-ɣ baseline cytokine expression in the normal and positive controls were 11.25 pg/ml and 30.64 pg/ml. After 24 hours of treatment with low and high doses of Vitamin D3, the IFN-ɣ cytokine expression were 21.53 pg/ml (P<0.01) and 16.62 pg/ml (P<0.001),

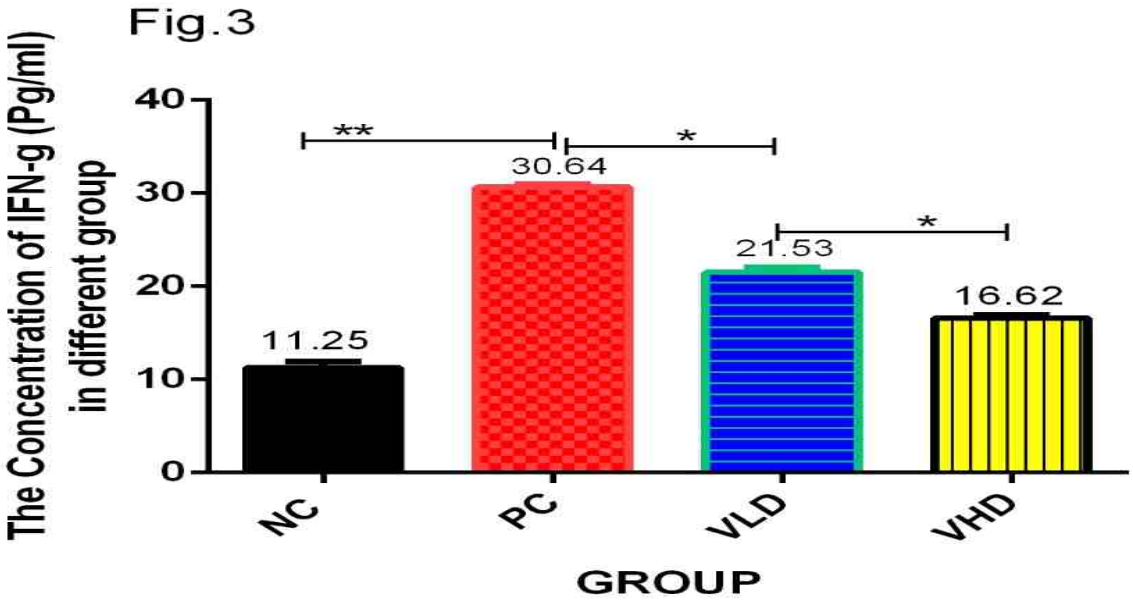

Figure: 3b, indicates an expression of IFN-γ concentrations in the controls and the treatment group. IFN-γ was significantly downregulated in VHD as compared PC.

Note: the PC is compared with the NC, and all the treatment groups are compared with the PC). P-value < 0.05 was considered statistically significant. (NC = Normal control, PC = Positive control, VLD = Vitamin D3 low dose, VHD = Vitamin D3 high dose, *P<0.01 **P<0.001, ***P<0.0001). All the data are representative of triplicate ELISA experiments.

The IL-6 Expression in Different Group

The IL-6 baseline cytokine expression in the normal and positive controls were 3.29 pg/ml and 16.55 pg/ml. After 24 hours of treatment with low and high doses of Vitamin D3, the IL-6 cytokine expression was 13.21 pg/ml(P<0.01) and 8.25pg/ml (P<0.001).

Fig. 4

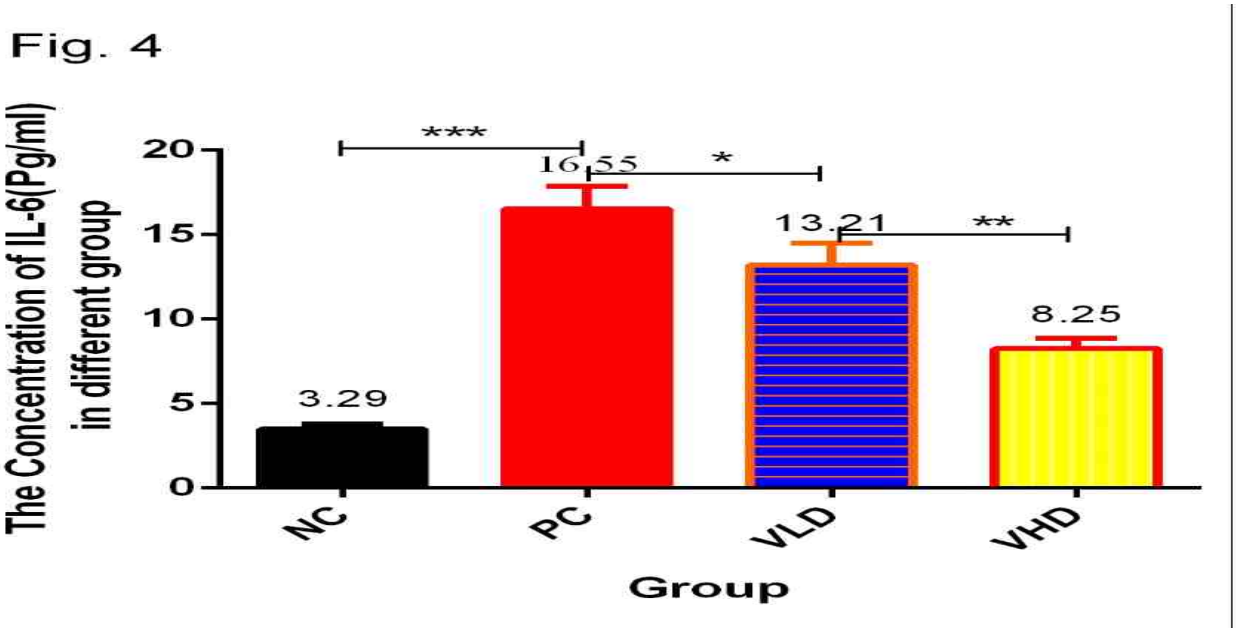

Figure 4: shows IL-6 cytokine concentrations in the controls and treated group. VHD was seen to be most important downregulator of IL-6 cytokine expression P=0.05.

Note: The PC is compared with the NC, and all the treatment groups are compared with the PC). P-value < 0.05 was considered statistically significant (NC = Normal control, PC = Positive control, VLD = Vitamin D3 low dose, VHD = Vitamin D3 high dose, *P<0.01 **P<0.001, ***P<0.0001). All the data are representative of triplicate ELISA experiments.

Discussion

New insights into the pathogenesis of IBD which focus on gene expression profile, has achieved tremendous success in the understanding of IBD treatment and management. Many studies have demonstrated overexpression of transcription factors and pro-inflammatory cytokines as the major causes of IBD (Xavier and Podolsky, 2007).

Th1 differentiation is regulated by T-bet transcription factor (T-box), referred to as the master regulator. T-bet possesses the ability to suppress and down-regulates the development of other T-cell subsets, such as Th2 and Th17 while facilitating, IFNγ production. T-bet expression is solely relied on signal transducer and activator of transcription 1 (STAT-1) and not on IL12 dependent STAT-4 (Calkins, 1989). Our research findings have indicated a threefold relative expression of T-bet gene in the positive controls, compared to the normal controls. This observation is in conformity with previous report which demonstrated that expression of T-bet is increased in CD patients and that T-bet regulates the mucosal cytokine balance in various murine experimental colitis models (Calkins, 1989). These observations may be due in part to the inhibition of Th2 by T-bet, which is initiated by constant and frequent suppression of IL-4 gene transcription, obstructing the activity of the Th2 master regulator, the GATA3. Thus, T-bet

initiates Th1 cell differentiation by activating Th1 and repressing Th2 genetic programmers (Cosnes *et al.,* 1999). This is a frequent occurrence in IBD, most especially in Crohn's disease. It was further observed in our study that after 24 hours of treatment and incubation with low and high doses of Vitamin D3, there was a relative down-regulation of T-bet gene expression when compared to the positive controls. This is in conformity with the report of Yasmin Morán-Auth et. al, who reported that STAT1 and T-bet gene expression levels were significantly inhibited by Vitamin D3 and another natural immunoregulatory in the cells of type 1 diabetes (T1D) and other autoimmune disease patients (Tysk *et al.,* 1988).

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. This increase is in agreement with the report which indicated that Crohn's disease shows a Th1 type of immune response with elevated proinflammatory cytokines, such as IL-12, TNF-α, and IFN-γ (Consortium, 2007). Our results have further shown that after 24 hours of treatment and incubation of patients' PBMC with Vitamin D3, there were significant down-regulations in the concentration of IFN-γ between the treated patients and positive controls PBMC. 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 (204). 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 (Lazareyic et al., 2011).

The IL-6, IL-11, IL-31 are members of the IL-6 family. The IL-6 / STAT3 signalling system plays an important role in CD pathogenesis. In this study, the baseline expression of IL-6 cytokine in the positive control was high compared to the positive control. This is in conformity with another research finding which reported a high circulating level of IL-6 and sIL-6R in IBD patient and this correlates with disease activity (Hata *et al.,* 2001). 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 (Hendrickson *et al.,* 2002). 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 very important in the pathogenesis of IBD and is most likely required for the extra-intestinal manifestation in IBD (Rogers *et al.,* 1971). Treatment of the PBMCs with various doses of Vitamin D3 has significantly downregulated the concentration of IL-6 in the treated groups as compared to the positive controls PBMC. This downregulation in IL-6 cytokine expression observed in this study could be attributed to the blockage of the maturation pathways of APCs, most especially, the dendritic cell by Vitamin D3 by constant inhibition of the actions of IL-12 and TNF-a 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 option in IBD treatment.

Conclusion

This research assessed the immunotherapeutic efficacy and potency of Vitamin D3 on IFN-γ, IL-6, and their transcription factors genes expression in human PBMC of patients with IBD. This research finding has further shed more light on the role of Vitamin D3 as a novel immunosuppressive, immunomodulatory, and non-steroidal anti-inflammatory drug in the management of IBD. Our research results have shown a pattern which indicates that Vitamin D3 seems to act in a dose-dependent manner, this is because their optimum activities were observed in all the groups treated with high doses of the drugs. It's worthy to note that the combination of half high doses of Vitamin D3 shows more immunosuppressive capacity and potency in all the proinflammatory genes and cytokines assessed. The drug have shown enormous potentials in immunomodulation and immunosuppression of all the pro and anti-inflammatory mediators that were assessed. We recommend that a well-designed, multi-centre

clinical trial be undertaken to determine the effects of Vitamin D3 in the overall management and treatment of IBD. Its clear from the result of this research that 1α,25(OH)2D3 is an immunosuppressive and immunomodulatory agent. It may be use in the management of IBD,

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