Vitamin D_3 Induced Decrease in IL-17 and Malondialdehyde, and Increase in IL-10 and Total Antioxidant Capacity Levels in Patients with Irritable Bowel Syndrome

Reza Amani[1,2], Amir Abbasnezhad[3,4*], Eskandar Hajiani[5], Bahman Cheraghian[6], Zahra Abdoli[7], Razieh Choghakhori[2]

[1]Diabetes Research Center, Health Research Institute, Department of Nutrition, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, [2]Food Security Research Center, Health Research Institute, Department of Clinical Nutrition, School of Nutrition and Food Science, Isfahan University of Medical Sciences, Isfahan, [3]Nutritional Health Research Center, Department of Nutrition, Lorestan University of Medical Sciences, Khorramabad, [4]Nutrition and Metabolic Diseases Researcher Center, Department of Nutrition, Ahvaz Jundishapur University of Medical Sciences, [5]Research Center for Infectious Diseases of the Digestive System, Ahvaz Jundishapur University of Medical Sciences,[6]Research Center for Infectious Diseases of Digestive System, Department of Biostatistics and Epidemiology, School of Public Health, [7]Department of Immunology, Faculty of medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

ABSTRACT

Background: Given the variations in clinical presentation and physiopathological mechanisms in irritable bowel syndrome (IBS) subtypes, it is an acknowledged fact that the response to treatments can be disparate. **Objective:** To assess the effect of vitamin D on inflammatory cytokines (IL-17, IL-10, TNF-α), and biomarkers of oxidative stress (total antioxidant capacity (TAC), and malondialdehyde (MDA)) among IBS patients. **Methods:** A double-blind, randomized, placebo-controlled 6-month intervention study was carried out on 90 IBS patients (85 were analyzed), as defined by the Rome III criteria. Study participants were randomly assigned to receive either 50,000 IU vitamin D_3 or a placebo fortnightly. **Results:** Vitamin D supplementation significantly reduced the IL-17 and MDA serum levels ($P<0.05$) and observably increased the TAC and IL-10 serum levels ($P<0.05$), compared with the placebo group. Comparing different bowel habit subtypes, we observed that it was only in diarrhea predominant IBS (IBS-D) that vitamin D supplementation was able to significantly reduce the serum levels of TNF-α and IL-17 ($P<0.05$). However, in all subtypes, IL-10 and TAC increased, while MDA decreased ($P<0.05$) in vitamin D group, compared to the placebo group. **Conclusion:** Vitamin D_3 supplementation reduces the serum IL-17 and MDA levels, and augments the serum IL-10 and TAC levels in IBS patients, particularly in IBS-D subtype. Thus, the present study demonstrates the beneficial effects of vitamin D on patients with IBS-D.

Amani R, et al. Iran J Immunol. 2018; 15(3): 186-196.

Keywords: Bowel Habit Subtypes, Inflammatory Cytokines, Irritable Bowel Syndrome, Oxidative Stress Biomarkers, Vitamin D

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*Corresponding author: Dr. Amir Abbasnezhad, Nutritional Health Research Center, Department of Nutrition, Lorestan University of Medical Sciences, Khorramabad, Iran, e-mail: abbasnezhad.amir@lums.ac.ir

INTRODUCTION

Irritable bowel syndrome (IBS) is a prevalent gastrointestinal (GI) disorder with a prevalence of 10%-25% worldwide (1). Based on predominant stool pattern, IBS is classified into diarrhea-predominant (IBS-D), constipation-predominant (IBS-C) and alternating (IBS-A), as defined by the Rome III criteria (2). Converging evidence suggests that there are several differences in perception, autonomic function and symptom characteristics between different bowel habit subtypes (3,4). Due to the heterogeneity of clinical symptoms and different pathophysiological mechanisms among subtypes of IBS (IBS-C, IBS-D and IBS-A), it is generally accepted that the response to treatments are different (5). The both central and peripheral factors are involved in the pathogenesis of IBS. Inflammation influenced gut functions, altering them in patients with IBS(6). An increase had been confirmed in the number of immune cells in the colon or terminal ileum of IBS patients via histological examination (6). Moreover, immune and inflammatory mediators such as pro-inflammatory cytokines were incriminated in the mechanisms which resulted in visceral hypersensitivity (7). Visceral hypersensitivity was incriminated in the development of clinical symptoms of IBS such as chronic discomfort and pain (7). Furthermore, in 2013, a study suggested that the disturbance in the oxidant-antioxidant balance might have a role in IBS and its clinical symptoms (8). The anti-inflammatory and immune-modulatory roles of vitamin D have been shown in several diseases (9,10). Vitamin D inhibited the proliferation and differentiation of mononuclear cells through reducing the production of pro-inflammatory cytokines (9). Vitamin D deficiency (VDD) and an increased risk of osteoporosis have been recently reported in IBS patients (11,12). A study demonstrated that vitamin D deficiency (VDD) might affect T helper cell (Th17) responses (13), and the normal levels of serum vitamin D protect against IL-17-mediated inflammation (13). Moreover, prior to this issue, the antioxidant role of vitamin D had been shown (14). To the extent of our knowledge, no clinical studies have evaluated the effect of vitamin D on biomarkers of inflammation or oxidative stress in patients with IBS.
In this study, we analyzed the effect of vitamin D on the inflammatory cytokines and the factors affecting oxidative stress in patients with IBS. Further evaluated was the effect of vitamin D on these factors in patients with different subtypes of IBS.

MATERIALS AND METHODS

Study Design and Population. A double-blinded, placebo-controlled randomized trial was conducted, with a parallel design, at Imam Hospital of Jundishapur University, Ahvaz, Iran during 6-month. Both female and male participants with IBS aged between 18 and 70 were enrolled according to the Rome III Diagnostic Criteria for IBS diagnosis in February and March 2015 (15). The subjects were randomly categorized into intervention and placebogroups (1:1 ratio). Patients visited the gastroenterologist at the baseline, after three months, and at the end of the trial. Patients were divided to different subtypes of diarrhea predominant IBS (IBS-D), constipation-predominant IBS (IBS-C), and alternating bowelhabits IBS (IBS-A). Details of the study design and population have been published recently (16). A gastroenterologist evaluated the patients for abdominal radiation or surgery, other GI disorders such as GI infection or celiac disease, diabetes, renal disorders, and any diagnosed or treated cancers, as exclusion criteria.

Additional exclusion criteria were pregnancy, lactation, alcohol consumption, hypercalcemia, special diet and consumption of vitamin E, omega-3, vitamin D and calcium supplements within 6 months prior to the study. The present study was approved by the Medical Ethics Committee at the Jundishapur University of Medical Sciences (Registration No. ir.ajums.rec.1394.306), and it was also registered at clinicaltrials.gov (NCT02579902). A written consent was obtained from all the participants.

Trial Interventions. In the control group, patients received a 50,000 IU pearl of vitamin D_3 orally every two weeks (Zahravi Pharm Co, Tabriz, Iran), for 6 months. Patients in the placebo group received one pearl of placebo (similar to the pearl of vitamin D_3) containing edible paraffin orally (Zahravi Pharm Co, Tabriz, Iran) every two weeks for 6 months.

Blood Sampling. The blood samples of the patients were obtained two times (before and after intervention) following an 8–12 h fasting. Five mL of blood was drawn from each sample. Serums were frozen immediately at -20ºC, and then stored at -80ºC.

Laboratory Analyses. All the laboratory analyses were conducted in the biochemical Laboratory of the Ahvaz Jundishapur University of Medical Sciences. The serum levels of 25(OH)D_3 and calcium were measured using radioimmunoassay method (Boldon, UK) and photometric test (Pars Azmoon Co, Tehran, Iran), respectively. Serum levels of 25(OH)D_3 ≥30 ng/mL were considered as sufficient, 20≤ to <30 ng/mL as insufficient, and <20 ng/mL as deficient (17). TNF-α, IL-10 and IL-17 were measured via an enzyme-linked immunosorbent assay method, with commercial reagents (BOSTER BIOLOGICAL TECHNOLOGY Co., Ltd., USA) according to the manufacturer's specifications. The serum malondialdehyde (MDA) and total antioxidant capacity (TAC) concentrations were measured using tiobarbituric acid and ferric reducing ability of plasma (FRAP) methods, respectively. Technicians were blinded to the intervention-placebo status.

Statistical Analysis. At first, the normal distribution of all variables was checked with the Kolmogorov–Smirnov test. The parametric and nonparametric data were reported as mean ± standard deviation (SD) or median (25th, 75th percentile), respectively. We compared the groups using Student's *t*-test, χ2-test, or Fisher's exact test, when appropriate. Using paired *t*-test, we further compared pre-and post-intervention biochemical factors in each group. The changes (endpoint minus baseline) of the two groups were compared using Independent *t*-test. One-way analysis of variance (ANOVA) was utilized for the comparison of the biochemical factors among different bowel habit subtypes of IBS. The differences between specific groups were analyze using Post-hoc analysis (Tukey's test). All data were analyzed using SPSS statistical software version 16 (SPSS Inc., Chicago, IL, USA). The differences with P-values <0.05 were considered as significant.

RESULTS

Baseline Characteristics of the Participants.

The flowchart of the subjects' allocation and their characteristics have been previously published (16). The mean age of the patients was 37.9 years old (ranging from 18 to 73 years), with more than two-thirds being females. At the baseline, bowel habit subtypes classification of IBS patients indicated that 40%, 25.9%, and 34.1% of patients were

IBS-A, IBS-D and IBS-C, respectively. As Table 1 indicates, no significant differences (P>0.05) were observed between the vitamin D and placebo groups in regard the baseline characteristics of the subjects.

Table 1. Baseline demographic and clinical characteristics in 85 patients with IBS.

All data are shown as mean ± standard deviation, and analyzed by two-sample t test unless otherwise indicated. † Data

Characteristics	Vitamin D (N=44)	Placebo (N=41)	*P* value
Age, years	37.45 ± 8.11	38.34 ± 9.85	0.65
Female †	28 (63.6)	29 (70.7)	0.49
IBS subtypes †			0.68
IBS-C	15 (34.1)	14 (34.1)	
IBS-D	13 (29.5)	9 (22)	
IBS-A	16 (36.4)	18 (43.9)	
Education level, n (%): †			0.62
None/ Primary	11 (25)	7 (17.1)	
Middle/ High school	19 (43.2)	18 (43.9)	
University or higher	14 (31.8)	16 (39)	
Smoking status, n (%): †			0.5
Never	32 (72.7)	27 (65.9)	
Smoking/ Ex-Smoker	12 (27.3)	14 (34.1)	
Duration of IBS symptoms†			0.57
1–5 years	12 (27.3)	9 (22)	
>5 years	32 (72.7)	32 (78)	
Family history of IBS †			0.64
Yes	14 (31.8)	15 (36.6)	
No	30 (68.2)	26 (63.4)	
BMI, kg/m^2	25.21 ± 2.72	24.98 ± 2.90	0.71
Body fat percentage	27.6 ± 6.08	28.24 ± 6.37	0.64
Dietary vitamin D intake (µg/d) ‡	0.00 (0.00, 1.34)	0.00 (0.00, 0.92)	0.74
Dietary calcium intake (mg/d)	611.23 ± 269.6	622.76 ± 317.43	0.86
Physical activity, MET-min/week	356.95 ± 273.53	324.42 ± 217.68	0.55

are numbers (%), and were analyzed by χ2 test or Fisher's exact test.‡ Data are median (25th, 75th percentiles), and tested by Mann–Whitney U test. IBS, irritable bowel syndrome; BMI, body mass index; MET, metabolic equivalent of task; IBS-C, constipation subtype; IBS-D, diarrhea subtype; IBS-A, alternating subtype; IBS-QOL, Irritable bowel syndrome quality of life; IBSSS, IBS severity score system.

Furthermore, the serum levels of $25(OH)D_3$, calcium, TNF-α, IL-10, IL-17, MDA and TAC were not different at the baseline between two groups (Table 2). Disease history and use of medication were comparable between the study groups. During and at the end of the study, no serious supplement-related adverse or side effects such as hypercalcemia were reported. A few minor back pains and headaches were reported in both vitamin D and placebo groups, which remained without any further manipulation. The rate of compliance in the intervention and placebo groups was 93.2 vs. 92.7 %, respectively.

Table 2. Biochemical variables of vitamin D and placebo groups at baseline.

TNF-α, tumor necrosis factor α; MDA, malondialdehyde; TAC, total antioxidant capacity; IL-10, interleukin 10; IL-17, interleukin 17. All data are analyzed by two-sample *t* test. *P* values <0.05 were considered statistically significant.

Variable	Vitamin D		Placebo		*P* value
	N=44		N=41		
	Mean	SD	Mean	SD	
Serum 25(OH)D_3, ng/mL	19.65	10.35	18.62	11.23	0.66
Serum calcium, mg/dL	9.03	0.30	8.96	0.32	0.31
Serum TNF-α, pg/mL	15.55	5.53	14.34	5.45	0.31
Serum IL-10, pg/mL	7.1	3.63	7.07	2.5	0.97
Serum IL-17, pg/mL	8.91	2.93	8.01	3.5	0.2
Serum MDA, ng/mL	3.87	1.54	3.8	1.55	0.81
Serum TAC, μmol/L	747.32	152.7	732.51	152.12	0.65

Biochemical Variables.

As expected, the serum levels of 25(OH)D_3 were significantly increased (not beyond the normal levels though) following supplementation with vitamin D vs. placebo group ($P<0.05$, Table 3). Based on intra-group comparison, the serum calcium levels did not change in either groups prior to and after intervention ($P>0.05$). Neither were any significant differences observed, post-intervention, in serum calcium between the two groups (Table 3). The serum levels of IL-17 and MDA were significantly decreased in the vitamin D group ($P<0.05$) vs. baseline (Table 3). Additionally, the serum levels of IL-10 and TAC increased significantly ($P<0.05$) in vitamin D group post-intervention. In the placebo group, IL-17, IL-10, TAC and MDA levels did not change significantly ($P>0.05$) at the end of the study. As Table 2 shows, in contrast to the placebo group ($P>0.05$), TNF-α levels were significantly reduced in the vitamin D group. There were no significant differences ($P>0.05$) between the two groups as far as TNF-α levels are concerned. However, a significant difference ($P<0.05$) was observed regarding the mean serum changes of IL-17, IL-10, MDA and TAC (Table 3).

Different Bowel Habit Subtypes.

As shown in Figure 1, following vitamin D supplementation, the serum levels of TNF-α (Mean changes: -3.15 ± 3.3 vs. -0.13 ± 2.7, $P<0.05$) and IL-17 (Mean changes: -4.8 ± 4.41 vs. -0.06 ± 5.6, $P<0.05$) were significantly decreased in patients with IBS-D, compared with the placebo. Following intervention, no observable differences were found in the mean changes of the TNF-α and IL-17 in either IBS-C (mean changes: -1.11 ± 2.3 vs. -0.62 ± 1.35, $P>0.05$; -4.18 ± 3.47 vs. -1.2 ± 4.33, $P>0.05$, respectively) or IBS-A patients (mean changes: -0.91 ± 3.7 vs. -0.81 ± 2, $P>0.05$; -4 ± 3.63 vs. -0.65 ± 5.66, $P>0.05$), as compared with placebo group (Figure 1).

Table 3. Within- and between-group comparisons of the changes from baseline to endpoint measures for serum biochemical variables in vitamin D and placebo groups of IBS patients.

Variable	Vitamin D N=44			Placebo N=41			*P* value [b]
	Value		*P* value [a]	Value		*P* value [a]	
	Mean	SD		Mean	SD		
Serum 25(OH)D_3, ng/mL							
6 mo	52.78	12.42	<0.001	20.91	10.93	0.07	
Change [c]	33.12	9.65		2.28	7.76		<0.001
Serum calcium, mg/dL							
6 mo	9	0.25	0.06	8.95	0.27	0.53	
Change	-0.03	0.10		-0.01	0.12		0.53
Serum TNF-α, pg/mL							
6 mo	13.91	5.06	0.002	13.74	5.08	0.052	
Change	-1.64	3.23		-0.59	1.91		0.07
Serum IL-10, pg/mL							
6 mo	16.28	10.01	<0.001	7.7	2.13	0.1	
Change	9.2	8.72		0.6	2.26		<0.001
Serum IL-17, pg/mL							
6 mo	4.62	2.28	<0.001	7.31	4.3	0.4	
Change	-4.3	3.75		-0.7	5.11		<0.001
Serum MDA, ng/mL							
6 mo	1.92	1.05	<0.001	3.46	1.51	0.12	
Change	-1.95	1.37		-0.34	1.4		<0.001
Serum TAC, μmol/L							
6 mo	1111.01	247.3	<0.001	777.92	177.7	0.8	
Change	363.7	245.93		45.41	164.5		<0.001

TNF-α, Tumor necrosis factor α; IL-10, interleukin 10; IL-17, interleukin 17; MDA, malondialdehyde; TAC, total antioxidant capacity.[a] *P* value for comparing baseline, with end point values within each group. Paired sample *t* test was used. *P* values <0.05 were considered statistically significant. [b] *P* value for comparing the changes of variables between the groups. Two-sample *t* test were used. *P* values <0.05 were considered statistically significant. [c] End—baseline.

In patients with IBS-C, IBS-D and IBS-A, the serum concentrations of the IL-10 (mean changes: 7.9 ± 10.1 vs. 0.31 ± 2.17,P<0.05; 11.84 ± 8.02 vs. 0.8 ± 1.71, P<0.05; 8.25 ± 7.91 vs. 0.74 ± 2.63, P<0.05, respectively) and TAC (mean changes: 351.17 ± 185.5 vs. 39.73 ± 110, P<0.05; 376.6 ± 309.4 vs. 54.31 ± 143.9, P<0.05; 364.94 ± 253.77 vs. 45.39 ± 211.2, P<0.05, respectively) significantly increased in vitamin D group (Figure 1). In addition, the serum MDA levels in IBS-C, IBS-D and IBS-A patients decreased significantly (mean changes: -1.84 ± 1.5 vs. -0.01 ± 1.7, P<0.05; -2.28 ± 1.34 vs. 0.1 ± 1,

P<0.05; -1.8 ± 1.32 vs. -0.7 ± 1.23, P<0.05, respectively) in vitamin D group vs. placebo group (Figure 1).

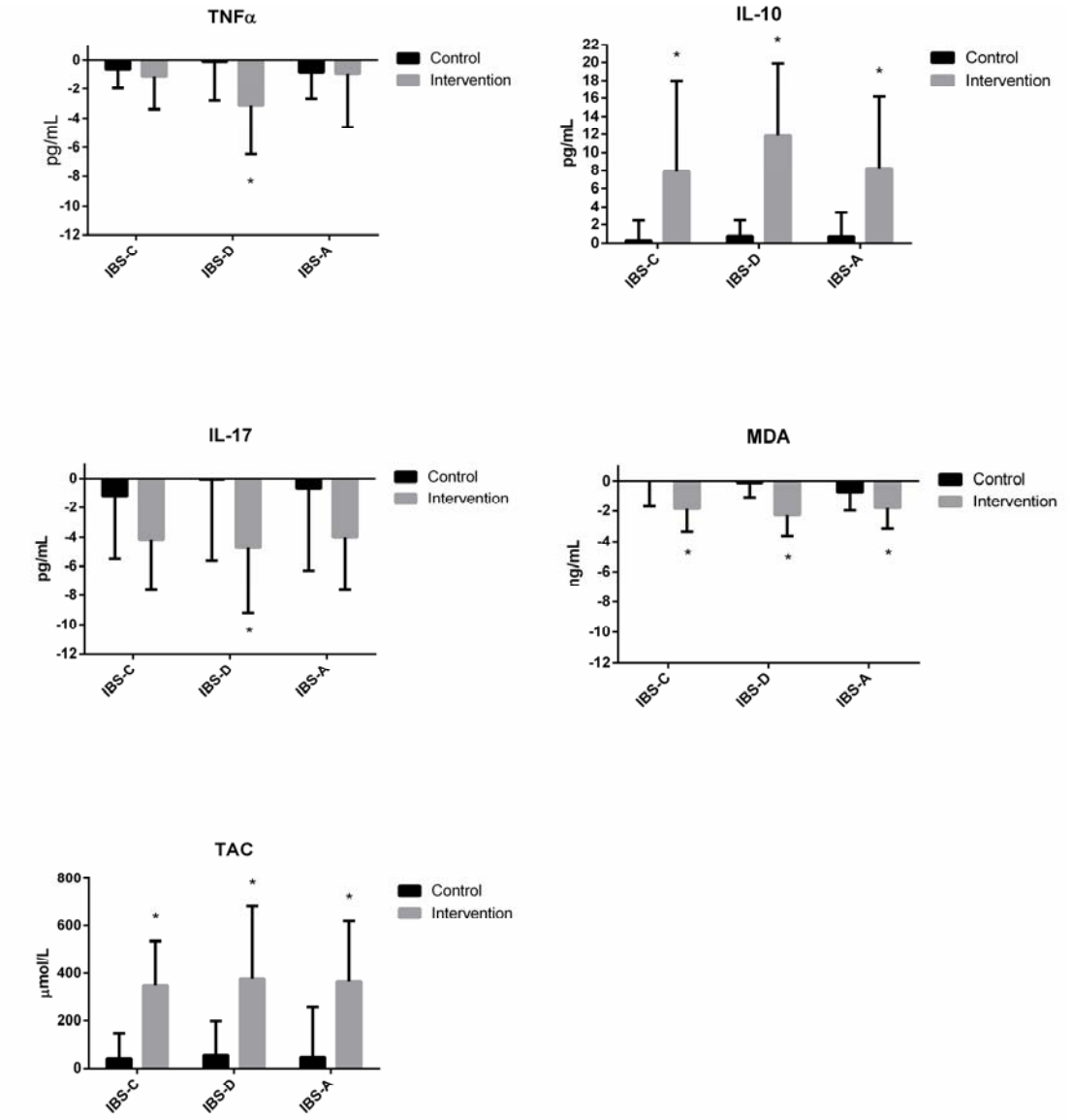

Figure 1. Mean ± SD changes of the serum levels of biochemical factors after 6-month in different bowel habit subtypes of IBS between vitamin D and placebo group. (* P<0.05), (number of the subjects: IBS-D=13, IBS-C=15, IBS-A=16, control=41).IBS, irritable bowel syndrome; MDA, malondialdehyde; TAC, total antioxidant capacity; IBS-D, diarrhea predominant IBS; IBS-C, constipation-predominant IBS; IBS-A, alternating bowelhabits IBS; SD, standard deviation.

DISCUSSION

The present randomized clinical study was carried out to evaluate the effect of vitamin D on IBS patients. After a 6-month supplement therapy with vitamin D_3 (50,000 IU), the significant (P<0.05) beneficial effects of vitamin D_3 were seen on both

inflammatory cytokines and antioxidant status. The findings of our study indicate that it was only in IBS-D patients that the serum TNF-α and IL-17 levels decreased following a 6-month vitamin D supplementation. In the present study, no adverse effects were reported in relation to the supplementation with vitamin D. A study reported the safety of doses of up to 10.000 IU/d in human (20). Furthermore, we have recently shown that 50,000 IU vitamin D administered fortnightly in patients with non-alcoholic fatty liver disease (NAFLD) has no such adverse effects as hypercalcemia. Following a four-monthvitamin D supplementation, vitamin D deficiency (<20 ng/mL) was not seen in the treated patients anymore (21). Moreover, in the study of Jalili *et al.* no adverse effects were associated with 50'000 IU vitamin D supplementation biweekly for six weeks in IBS patients (22). Therefore, we prescribed 50,000 IU vitamin D every two weeks to augment the effect of suboptimal serum 25(OH)D levels. The pathogenesis of IBS has not been fully known. As previously indicated, both peripheral and central factors are responsible in the pathogenesis of IBS (6). It has been shown that pro-inflammatory cytokines (such as TNF-α, IL-1β and IL-6) have higher rates in IBS patients compared with healthy subjects (23). Recent findings suggest that inflammation has an important role in altering gut function and visceral hypersensitivity in IBS (6). Inflammation and any injury to tissues are involved in the mechanism that leads to a heightened sensitivity of nervous system, thereby causing an enhanced pain sensitivity, a phenomenon known as visceral hypersensitivity (24,25). Furthermore, research has revealed that the autonomic dysfunction and the exaggerated hypothalamo-pituitary-adrenal (HPA) axis response to stressors, which is the key endocrine stress system in humans, are correlated with inflammation in IBS patients (26). It has been demonstrated that circulating pro-inflammatory cytokines like TNF-α, regulate the secretion of the corticotrophin-releasing hormone (CRH), which is the primary hypothalamic regulatory peptide of HPA axis (27). It has been well documented that vitamin D is potentially an immunomodulatory and anti-inflammatory factor able to influence inflammatory cytokines in several diseases (28-30). In the study of Schleithoff *et al.*(29) after a 9-month intervention with 50μg/d vitamin D_3 plus 500 mg/d Ca, median IL-10 concentrations increased, while TNF-α remained unchanged when compared with placebo plus 500 mg/d Ca group. Furthermore, in patients with type 2 diabetes, the serum IL-10 concentrations increased significantly ($P<0.05$) upon receiving vitamin D fortified drink, as compared with those receiving an unfortified drink (30). In yet another study, following a biweekly vitamin D_3 (50,000 IU) intervention for 4 months in NAFLD patients, serum TNF-α concentration did not change significantly ($P>0.05$) when compared with the placebo group (21). Similarly, the present study was unable to identify any significant differences in the mean change of TNF-α levels between the groups; the serum IL-10 concentrations, on the other hand, increased following supplementation with vitamin D (Table 3). IL-17 is a potent mediator of inflammation, inducing several inflammatory genes (31); thus, for the future pharmacotherapy, IL-17 family members and their receptors, are potential targets (32). The current study found that the serum IL-17 levels decreased after six months of intervention with vitamin D, and remained unchanged in the placebo group. The impact of vitamin D on IL-17 production by T cells was assessed in the study of Correale *et al*. (33), where they found that $1,25(OH)_2D$ reduced the number of IL-17 producing T cells and increased IL-10 producing cells. In another study, $1,25(OH)_2D_3$ had a potential role in modulating the capacity of CD4+T cells in response to allergens and down-regulated the allergen-induced expression of IL-13 and IL-17 (34).Furthermore, evidence suggests that

oxidative stress may be involved in the pathogenesis of IBS (8), by causing tissue injury, and activating immune cells, which consequently lead to visceral hypersensitivity (8,24). The antioxidant role of vitamin D has been previously indicated in other diseases (14,21). Asemi *et al*. revealed that the administration of 400 IU/d cholecalciferol for 9 weeks significantly ($P < 0.05$) increased TAC concentration among pregnant women (35). Moreover, following 300,000 IU vitamin D_3/month supplementation for 3 months in asymptomatic vitamin D-deficient subjects, the serum levels of MDA significantly decreased ($P<0.05$) (36). Similarly, we found that vitamin D was able to significantly ($P<0.05$) augment the mean serum levels of TAC, and decrease MDA in IBS patients. The results related to the comparison of different bowel habit subtypes demonstrated that vitamin D supplementation could decrease the pro-inflammatory cytokines (TNF-α and IL-17) in patients with IBS-D, while no significant changes ($P>0.05$) were seen in other subtypes. However, in all subtypes, the serum levels of anti-inflammatory cytokine IL-10 increased in vitamin D group compared with the placebo. No observable difference ($P>0.05$) was observed between the subtypes concerning the mean changes of oxidative stress biomarkers (MDA and TAC) following vitamin D therapy. The explanations associated with different responses in different IBS subtypes to a treatment, are multifactorial and include pathophysiological differences (3,4). Recent studies have demonstrated that the serum inflammatory cytokine levels are different between bowel habit subtypes (23,37). Liebregts *et al.* demonstrated that the release of inflammatory cytokines such as TNF-α, IL-1β, IL-6, increased in IBS-D patients, an augmentation associated with IBS symptoms (23). Moreover, Hughes *et al.* demonstrated that the serum inflammatory cytokine levels were higher in patients with IBS-D (38), and that the serum TNF-α levels correlated positively with the self-reported pain intensity in IBS-D patients, in contrast to IBS-C patients (38). A limitation in the present study is that the psychological factors such as anxiety and depression were not evaluated in patients. Moreover, tiobarbituric acid method was employed for measuring serum MDA levels, which entails the specificity and variability of data. In order to overcome this issue, it is possible to directly measure MDA via UV absorption with HPLC. The recruitment of patients from an outpatient clinic further curbed generalizability of our results. All said, our study was the first to assess the effect of vitamin D on inflammatory cytokines and oxidative stress on IBS patients. Low-grade mucosal inflammation and increased immune activityin IBS play major roles in altered gut function, visceral hypersensitivity, and clinical presentation of IBS (6). Moreover, disturbances in pro-oxidant-antioxidant balance have been reported to be involved in the pathogenesis of IBS (8). The authors identified anti-inflammatory and antioxidant effects of vitamin D in IBS patients based on the aforementioned notion. Furthermore, vitamin D supplementation was able to reduce pro-inflammatory cytokines in IBS-D patients.Our study points to the beneficial effects of vitamin D on patients with IBS-D; nevertheless, more research is required to further support the use of vitamin D in clinical settings.

ACKNOWLEDGEMENTS

This work was financially supported by a Grant (No. NRC-9410) from the Vice-Chancellor for Research at the Jundishapur University of Medical Sciences and

approved by the Nutrition and Metabolic Diseases Research Center, Jundishapur University of Medical Sciences, Ahvaz, Iran.

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