Tropical Journal of Pharmaceutical Research April 2024; 23 (4): 747-752
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v23i4.10

Original Research Article

Effect of 1, 25-dihydroxyvitamin D3 on T-Helper 17 (TH17) cell pathways in diarrhea-associated irritable bowel syndrome

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Sent for review: 22 November 2023 *Revised accepted: 23 March 2024*

Abstract

Purpose: *To investigate the effect of 1, 25-dihydroxyvitamin D3 (1,25-(OH)2D3) on T-Helper 17 (TH17) cell pathways in diarrhea-associated irritable bowel syndrome (IBS-D).*
Methods: *120 children with IBS-D were treated with Smecta alone and Smecta in addition to 1,25-(OH)2D3 in Hangzhou Ninth Peoples Hospital, Hangzhou, China. Blood samples were taken to determine the levels of 1,25-(OH)2D3, IL-17, IFN-γ, IL-23, and IL-6, as well as Th17 cell ratio. Furthermore, IL-17 and RORγt expressions were evaluated.*
Results: *Serum 1,25-(OH)2D3 levels decreased in IBS-D patients and were negatively correlated with IL-17 levels in serum and Th17 cells in peripheral blood. Treatment with 1,25-(OH)2D3 significantly reduced serum IFN-γ, IL-17, IL-23 and IL-6 levels in IBS-D patients ($p < 0.05$). Percentage of Th17 cells, and IL-17 and RORγt expression levels were significantly reduced in IBS-D patients after 1,25-(OH)2D3 treatment ($p < 0.05$).*
Conclusion: *This study shows that 1,25-(OH)2D3 reduces serum IFN-γ, IL-17, IL-23, IL-6 levels, Th17 cells and RORγt expression in IBS-D patients. Additional studies with larger sample sizes are required to validate these findings.*

Keywords: *Diarrhea irritable bowel syndrome, 1,25-Dihydroxyvitamin D3, Th17 cells, IL-17, RORγt*

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INTRODUCTION

Diarrhea-associated irritable bowel syndrome (IBS-D) is a common intestinal disorder. Statistically, about 10 - 20 % of global population is affected by IBS-D, with children and adolescents being the most vulnerable patient groups [1]. Prevalence of IBS-D varies depending on population. To date, prevalence of IBS-D in children has increased annually in recent years [2]. Overall, IBS-D has become a public health problem that must be addressed urgently. The mechanism of IBS-D is still unclear, but studies have revealed that the number of Helper T-cells (Th17) in the intestines of IBS-D patients increases, resulting in the enhancement of mucosal inflammatory responses and intestinal allergic responses [3]. This subsequently leads to symptoms such as disturbed intestinal motor function, abdominal

pain and diarrhea. Therefore, regulation targeting Th17 cells may be a therapeutic option for IBS-D treatment. 1,25-dihydroxyvitamin D3 (1,25-(OH)2D3) has been shown to modulate the immune and nervous systems, influence the composition of intestinal microbiota, uphold intestinal flora stability, and enhance intestinal development and integrity [4,5]. In addition, 1,25-(OH) 2D3 was found to inhibit Th17 cell development to elicit immunomodulatory response [6]. Recent studies demonstrate that patients with IBS-D are frequently deficient in 1,25-(OH)2D3, and its supplementation may improve symptoms [7]. However, there is still a lack of large-scale randomized controlled experiments to verify the therapeutic effect of 1,25-(OH)2D3 for IBS-D. Therefore, this study investigated the efficacy of 1,25-(OH)2D3 on Th17 cells and related cytokine production in children with IBS-D.

METHODS

Subjects

A total of 120 children treated with IBS-D at Hangzhou Ninth Peoples Hospital were randomly divided into IBS-D + Smecta (received Smecta) and IBS-D + Smecta + 1,25 (OH) 2D3 (received 1,25 (OH) 2D3 in addition to Smecta) groups comprising of 60 patients each. A total of 40 healthy children were used as controls. This study was approved by the Ethics Committee of Hangzhou Ninth Peoples Hospital (approval no. 2022-061) and conducted following the guidelines of Declaration of Helsinki [8]. All the guardians signed written informed consent.

Sample collection

Peripheral blood samples (5 mL) were collected and centrifuged for 10 min at 3000 rpm. Centrifugation with Ficoll-Hypaque density gradients was employed to isolate peripheral blood mononuclear cells (PBMCs). Furthermore, PBMCs were exposed to 0, 10 and 100 nmol/L of 1,25- (OH) 2D3, respectively, and then collected for further analysis.

Enzyme-linked immunosorbent assay (ELISA)

Beyotime's ELISA kits were employed for quantification of serum 1,25-(OH)2D3, Interleukin 17 (IL-17), Interferon-γ (IFN-γ), IL-23 and IL-6 concentrations.

Flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood and pretreated with phorbol 12-myristate 13-acetate (PMA) (25 ng/mL), ionomycin (50 µg/mL) and monensin (50 mg/mL) for 4 h at 37 ℃. Thereafter, the PBMCs were stained with specific antibodies to mark the expression of Th17 cell surface molecule. Finally, the Th17 cells were examined using flow cytometry (Agilent, Beijing, China).

Quantitative real-time PCR (qRT-PCR)

A TRIzol (Invitrogen, Carlsbad, USA) extraction of RNA was performed on the PBMCs and prepared for cDNA using QuantiTect RT kit (QIAGEN, Hilden, Germany). Subsequently, qRT-PCR was employed to measure mRNA levels of IL-17 and RORγt with SYBR Green mix from Takara (Dalian, China) on the ABI7500 system. The primer sequences used are presented in Table 1. Relative mRNA expression was calculated by the $2^{-\Delta\Delta ct}$ method.

Table 1: Primer sequences used for qRT-PCR

Gene	Forward (5'-3')	Reverse (5'-3')
IL-17	CCCCTAGACTCA GGCTTCCT	AGTTCGTTCTGC CCCATCAG
RORγt	AGTGTAATGTGG CCTACTCCT	GCTGCTGTTGCA GTTGTTTCT
β-actin	CTTCGCGGGCGA CGAT	CCACATAGGAAT CCTTCTGACC

Western blot

The PBMCs were lysed with radioimmuno-precipitation analysis (RIPA) buffer (Invitrogen). Then, 12 % sodium dodecyl sulphate acrylamide gel electrophoresis (SDS-PAGE) was used, and the proteins were transferred using poly(vinylidene fluoride) (PVDF) membrane. Primary antibodies against RORγt (ab135669, 1:1000, Abcam) andβ-actin (ab8227, 1:1000, Abcam) were incubated with the membranes, and then secondary antibodies were added. The bands were visualized and quantified.

Statistical analysis

The data were analyzed using Statistical Packages for Social Sciences (IBM, Armonk, NY, USA). Categorical data was presented as mean ± standard deviation (SD) and compared with Student's t-tests (unpaired) and one-way ANOVA (multiple comparisons). Figures were prepared using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA). Correlation was calculated using Pearson's correlation coefficient. $P < 0.05$ was considered statistically significant.

RESULTS

Serum 1,25-(OH)2D3, Th17 cells and IL-17 levels

The results of ELISA showed that 1,25-(OH)2D3 level significantly decreased, whereas IL-17 increased compared to control (CTRL) group (Figure 1 A and B). Also, the results of flow cytometry showed a significantly increased ratio of Th17 in IBS-D group compared to CTRL group (Figure 1 C). Moreover, correlation analyses showed inverse correlations between serum levels of 1,25-(OH) 2D3 and IL-17 (Figure 1 D), as well as and negative correlation with Th17 cell proportions (Figure 1 E). Also, there was an association between 1,25-(OH)2D3 deficiency and excessive activation of Th17 cells in IBS-D patients.

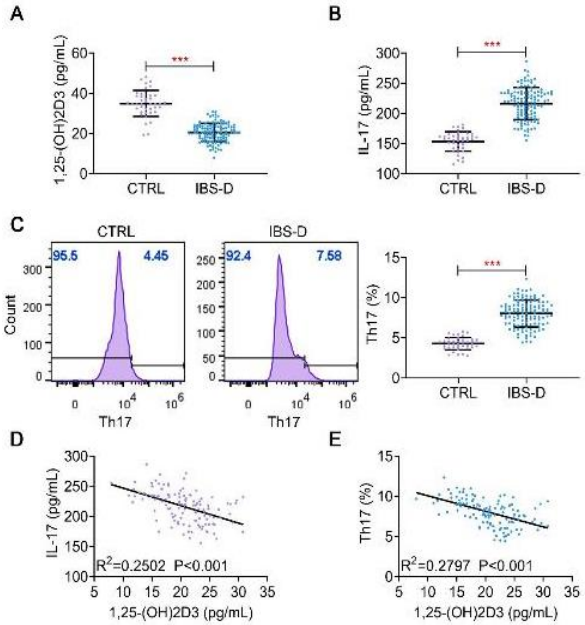

Figure 1: Correlation of serum 1,25-(OH) 2D3, Th17 cells and IL-17 levels from IBS-D patients. (A & B) Serum 1,25-(OH) 2D3 and IL-17 levels were examined by ELISA; (C) Proportion of Th17 cells in peripheral blood was analyzed by flow cytometry; (D) Pearson correlation between 1,25-(OH) 2D3 and IL-17 levels; (E) Pearson correlation between 1,25-(OH) 2D3 level and Th17 cells. ***P < 0.001 vs. CTRL group

Effect of 1,25-(OH)2D3 on serum immune indices

There was no significant difference in serum IFN-γ (Figure 2 A), IL-17 (Figure 2 B), IL-23 (Figure 2 C) and IL-6 (Figure 2 D) levels in IBS-D + Smecta and IBS-D + Smecta + 1,25-(OH)2D3 groups. After treatment, serum from IBS-D + Smecta + 1,25-(OH) 2D3 group patients showed significantly lower levels of these indexes compared to the IBS-D + Smecta group. This suggests that 1,25-(OH) 2D3 inhibits immune response in IBS-D patients.

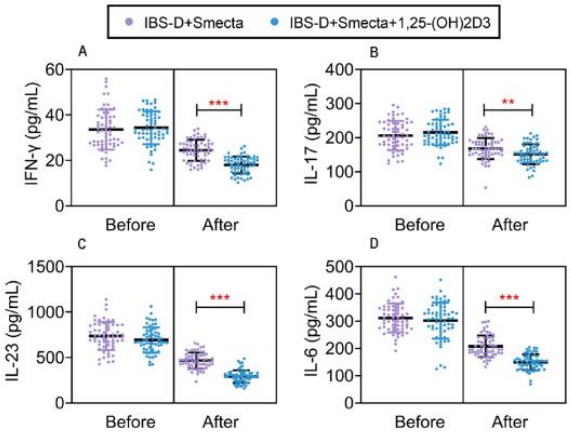

Figure 2: Effects of 1,25-(OH)2D3 serum immune indices. (A) Serum IFN-γ levels, (B) Serum IL-17 levels (C) Serum IL-23 levels (D) Serum IL-6 levels were all determined by ELISA. **P < 0.01, ***p < 0.001 vs. CTRL group

1,25-(OH)2D3 exerts immunomodulatory effect on Th17 cells in IBS-D patients

1,25-(OH) 2D3 was administered to PBMCs to study its immune-regulatory effect on Th17 cells. The flow cytometry results showed that 1,25-(OH) 2D3 (10 and 100 nmol/L) treatment decreased Th17 cell ratio in PBMCs (Figure 3 A and B). Also, the results of qRT-PCR showed that IL-17 mRNA significantly reduced after 1,25-(OH) 2D3 (10 and 100 nmol/L) treatment (p < 0.05) (Figure 3 C). Thus, 1,25-(OH) 2D3 exerted immunomodulatory roles on Th17 cells in IBS-D patients.

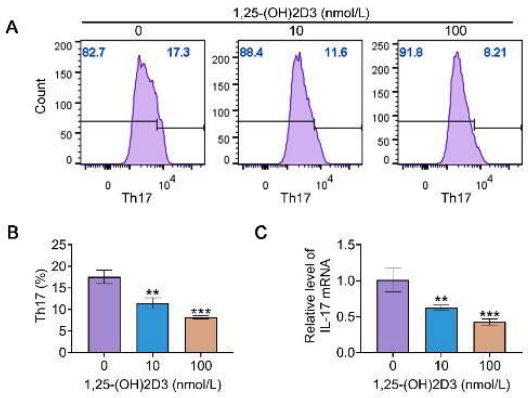

Figure 3: 1,25-(OH) 2D3 exerts immunomodulatory effect on Th17 cells in IBS-D patients. (A-B) The percentages of Th17 cells in peripheral blood were analyzed by flow cytometry; (C) The mRNA level of IL-17 was determined by qRT-PCR. **P < 0.01, ***p < 0.001 vs. CTRL group

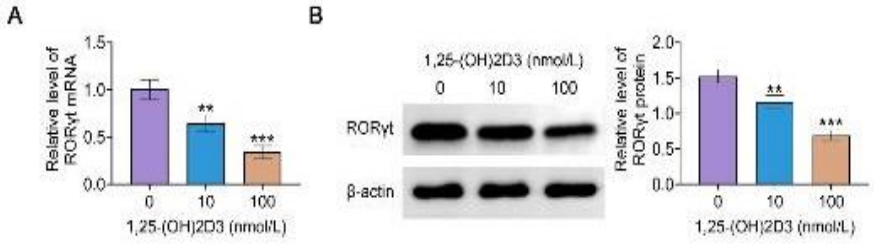

Figure 4: 1,25-(OH) 2D3 suppresses expression of RORγt in peripheral blood. (A) The mRNA level of RORγt was determined by qRT-PCR; (B) Protein level of RORγt was determined by western blot. ***P* < 0.01, ****p* < 0.001 vs. CTRL group

1,25-(OH)2D3 suppresses RORγt expression in peripheral blood

Further analysis was conducted to evaluate the effect of 1,25-(OH) 2D3 on RORγt expression. Based on qRT-PCR and western blot analyses, RORγt mRNA and protein levels were significantly reduced in 1,25-(OH) 2D3 (10 and 100 nmol/L) treatment groups when compared to control group (Figure 4 A, 4 B). Thus, 1,25-(OH) 2D3 inhibits RORγt expression in peripheral blood from IBS-D patients.

DISCUSSION

Patients with IBS-D often experience abdominal pain which adversely affects their quality of life and psychology [9]. With the evolution of intestinal microecology, studies have shown that IBS-D is largely caused by changes in gut microbiota [10]. Th17 cells are a type of T lymphocyte that plays a proinflammatory role in immune response by producing and secreting IL-17 [11]. Evidence showed that abnormal activation and aggregation of Th17 cells in gut mucosa of IBS-D patients disrupts intestinal mucosal barrier, and promotes intestinal flora disorders and immune cell activation, which lead to an inflammatory and intestinal allergic response, causing diarrhea, abdominal pain, bloating and other symptoms [12]. Therefore, therapeutic options targeting Th17 cells offer promising new directions for IBS-D treatment. 1,25(OH) 2D3 has been shown to play a vital role in intestinal health and immune adjustment, and is essential for the maintenance of intestinal epithelial barrier [13].

Studies have found that reduced 1,25(OH) 2D3 levels are associated with intestinal inflammation and microbiota dysbiosis in IBS-D patients [14]. Furthermore, 1,25(OH) 2D3 alleviates intestinal inflammation and reduces the production of proinflammatory mediators by inhibiting T cell-mediated immune response [15]. This study revealed lower 1,25(OH) 2D3 levels, higher IL-17 levels, and increased Th17 cells in the peripheral blood of IBS-D patients, with a negative relationship between 1,25-(OH) 2D3 and IL-17 or Th17 cells. This suggests that 1,25-(OH) 2D3 deficiency is linked to excessive Th17 cell activation in IBS-D patients. Previous studies have found that 1,25-(OH) 2D3 acts as an immunomodulator by inhibiting Th17 cell development and function. For example, in kidney transplant recipients, 1,25-(OH) 2D3 effectively suppressed Th17-related immune responses [16]. 1,25-(OH) 2D3 was intended to regulate Th17-mediated immunity by inhibiting Th17 cells and expression of related factors in systemic lupus erythematosus [6].

This study showed that serum IFN-γ, IL-17, IL-23 and IL-6 levels were decreased in IBS-D patients treated with 1,25-(OH) 2D3, indicating that 1,25-(OH) 2D3 modulates Th17-associated pathways to exert an immunomodulatory effect in IBS-D. The RORγt, which is a key transcription factor, promotes the differentiation and function of Th17 cells [17]. It has been demonstrated that RORγt effectively promotes the production and secretion of IL-17 and IL-23 during Th17 cell differentiation [18]. In addition, it also regulates T cell distribution in intestinal and mucosal tissues, thus affecting immune homeostasis and gut function [19]. This study revealed that RORγt mRNA and protein were significantly reduced after 1,25-(OH) 2D3 treatment. This suggests that 1,25-(OH) 2D3 may be beneficial in improving IBS-D by disturbing RORγt signaling pathway to suppress Th17 cell differentiation.

Limitations of this study

The study is limited by its small sample size; as a result, the effect of 1, 25-dihydroxyvitamin D3 on T-Helper 17 cells may not have been fully investigated.

CONCLUSION

The compound, 1,25-(OH)2D3, reduces serum IFN-γ, IL-17, IL-23, IL-6 levels, and Th17 cells and RORγt expressions in the peripheral blood of

children with IBS-D, providing a new theoretical basis and therapeutic options for the treatment of IBS-D. Further studies with larger sample sizes will be required to validate the findings in this study.

DECLARATIONS

Acknowledgements

This work was supported by Hangzhou Medical Health Science and Technology Project (grant no. 20220919Y070).

Funding

None provided.

Ethical approval

This study was approved by the Ethics Committee of Hangzhou Ninth Peoples Hospital (approval no. 2022-061).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Yongjian Li and Yan Wang designed the study and carried them out, supervised the data collection, analyzed and interpreted the data, prepared the manuscript for publication and reviewed the draft of the manuscript. All authors read and approved the manuscript for publication.

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