Effects of vitamin D supplementation on red cell indices and cytokines in patients with thalassemia: An open-label randomized clinical trial

Raz Muhammed HamaSalih*, Marwan S.M. Al-Nimer|*****
***Department of Pharmacology and Toxicology, College of Pharmacy, University of Sulaimani**
**** Clinical Pharmacology and Therapeutics,College of Pharmacy, Hawler Medical University, Erbil, Iraq.**
***** Clinical Pharmacology and Therapeutics, School of Medicine, Faculty of Medical Sciences, University of Sulaimani, Sulaimani, Iraq.**

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Corresponding Author email:
raz.hamasalih@univsul.edu.iq
orcid: https://orcid.org/0000-0001-7139-6207

Abstract:

Background and aim
Deficiency of vitamin D is known as a health problem all over the world and a recognized clinical complication of beta thalassemia patients. Vitamin D acts as a hormone at the nuclear receptor rendering it a beneficial medication for a number of diseases. It is believed that vitamin D is important in the modulation of the inflammation system by regulating the formation of inflammatory cytokines and immune cells. This study aimed to investigate the effect of vitamin D supplementation on the red cell indices and cytokines levels in patients with beta thalassemia major, in an open label randomized clinical trial.

Patients and Methods: this study performed an open-label randomized clinical trial in patients with beta thalassemia major. Forty-six patients completed the eight weeks clinical trial and were allocated to administer oral vitamin D3 supplement of 100,000 IU every two weeks as an add-on treatment. During the study, hematological indices, serum iron, ferritin, vitamin D, calcium and inflammatory markers (interleukin-6, interleukin-2 and interleukin-10) were evaluated before (at baseline) and after vitamin D supplementation for 8 weeks.

Results: Vitamin D3 supplements significantly decreases interleukin-6 levels and elevates the serum levels of anti-inflammatory cytokines IL-2 and IL-10, it also significantly reduced serum ferritin level, but it did not alter the hematological indices.

Conclusion:
Our results suggest that administration of vitamin D has a potential anti-inflammatory role in beta thalassemia patients and reduces serum ferritin levels, which may reduce the burdens of iron overload in thalassemia patients.

Key words: Beta thalassemia major, vitamin D3, inflammation and interleukin

آثار مكملات فيتامين (D) على مؤشرات الخلايا الحمراء ومستويات السيتوكينات في المرضى الذين يعانون من ثلاسيميا بيتا الرئيسية; تجربة سريرية عشوائية ذات علامة مفتوحة.

*راز محمد حمه صالح،** مروان صالح النمر

الخلاصة:
خلفية:يعرف نقص فيتامين (D) بأنه مشكلة صحية في جميع أنحاء العالم ومضاعفات سريرية معترف بها لمرضى الثلاسيميا بيتا. يعمل فيتامين (D) كهرمون في المستقبل النووي مما يجعله دواء مفيد لعدد من الأمراض. ويعتقد أن فيتامين (D) مهم في تعديل نظام الالتهابات من خلال تنظيم تشكيل السيتوكينات الالتهابية والخلايا المناعية.
الهدف :
تهدف هذه الدراسة إلى دراسة آثار مكملات فيتامين (D) على مؤشرات الخلايا الحمراء ومستويات السيتوكينات في المرضى الذين يعانون من ثلاسيميا بيتا الرئيسية، في تجربة سريرية عشوائية ذات علامة مفتوحة.
الإعداد: مركز الثلاسيميا ، دليل الصحة ، المستشفى العام فى السليمانية، إقليم كوردستان - العراق.
الطريقة:
أجرينا تجربة سريرية عشوائية مفتوحة التسمية على المرضى الذين يعانون من ثلاسيميا بيتا الرئيسية. أكمل ستة وأربعون مريضا التجربة السريرية (الكلينيكية) التي استمرت ثمانية أسابيع وتم تخصيصها لإعطاء مكملات فيتامينD3 عن طريق الفم والتي تبلغ ١٠٠٠٠٠ وحدة دولية IU كل أسبوعين كعلاج إضافي. خلال الدراسة، تم تقييم مؤشرات الدم، الحديد في الدم، فيريتين، فيتامين D ، الكالسيوم وعلامات الالتهابات (إنترلوكين -٦، إنترلوكين -٢ وإنترلوكين -١٠) قبل (في الأساس) وبعد مكملات فيتامين (D) لمدة ثمانية أسابيع.
النتائج :
مكملات فيتامين D3 تقلل بشكل كبير مستويات إنترلوكين -٦ وترفع مستويات مصل السيتوكينات المضادة للالتهابات-IL 2و10-IL ، كما أنه يقلل بشكل كبير من مستوى فيريتين المصل، لكنه لم يغير مؤشرات الدم.
خاتمة:
تشير نتائجنا إلى أن تناول فيتامين (D) له دور محتمل مضاد للالتهابات في مرضى الثلاسيميا بيتا ويقلل من مستويات فيريتين المصل، مما قد يقلل من أعباء الحديد الزائدة في مرضى الثلاسيميا.
الكلمات المفتاحية: بيتا ثلاسيميا الكبرى ، وفيتامين D3 ، والالتهاب وانترلوكين.

Introduction

Thalassemia is a hemoglobinopathy that is inherited and induced by omission of beta globin chain (beta-thalassemia) or alpha chain (alpha thalassemia) [1]. Thalassemia patients are typically treated with frequent blood transfusions used in combination with iron chelating drugs such as deferiprone, desferroxamine [2]. Iron excess in tissues is the primary complication of thalassemia. Latest studies emphasize relationship of thalassemia with vitamin D deficiency. Several studies explain the advantage of utilizing vitamin D as add-on therapy in many illnesses [3-5]. Additionally, a variety of chronic diseases have been reported to have reduced level of vitamin D and there is high occurrance of vitamin D deficiency in thalassemic children [6-8]. Serum level of vitamin D should be assessed frequently in all children and older people with thalassemia [9]. Deficiency of vitamin D is common in children with beta-thalassemia [10-11]. Furthermore, there is a connection between vitamin D deficiency and hepatic iron excess as a result of impairment of 25 OH-hydroxylase enzyme activity in the liver [12]. There are numerous independent risk factors that are in charge of reduced vitamin D level in thalassemia patients such as serum ferritin and hepatic iron overload [13]. Vitamin D is discussed by many authors to have other health benefits besides its positive effects on bone, such as reducing inflammation [14-16]. Observational studies have also reported that higher vitamin D levels were associated with lower inflammatory markers such as IL-6 [17]. Elevation of serum vitamin D levels in patients with thalassemia can cause alterations in the serum iron or in the red cell indices that are related to thalassemia.
In this study we studied the effect of vitamin D supplementation on the red cell indices and cytokines levels beta thalassemia major patients.

Patients and methods

Study design

This study performed a prospective, randomized, open-label clinical trial between February 2018 and July 2018 at the Center of Thalassemia, Directory of Health, General Hospital Sulaimani. Demographic data including age, gender, body weight, height, and medical history was recorded by thorough history from parents/guardians and from patient's medical record. All the

patients were treated with the same therapeutic iron chelating regimen *plus* vitamin D3 oral ampoule as an add-on-therapy 100,000 IU/every two weeks (Dibase, 100,000 IU/ml solution for intramuscular and oral use, Abiogen Pharma, Pisa-Italy) for eight weeks. The patients were regularly transfused monthly and were being treated with iron chelating agents. The supplement used by the patients was harmless and without adverse reactions, and the patients were allowed to withdraw from the study at any time he/she wished.

Ethical approval:

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee [Clinical Research Ethics Committee of College of Medicine, University of Sulaimani (certificate no 7/29/3143) according to the guidelines that fulfill the requirement of postgraduate research and the International Continuous Medical Education] and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Participants

Fifty patients were recruited for the study design; four patients were lost in follow up, a total of forty-six patients were included in the final data analysis. Patients were enrolled when they fulfill the diagnostic criteria for thalassemia based on the hemoglobin electrophoresis in which beta thalassemia trait usually has reduced or absent HbA, elevated levels of HbA2, and increased HbF [18]. The criteria of inclusion were transfusion-dependent children and adolescents with beta-thalassemia major not receiving vitamin D containing supplements aged between 6-18 years. The criteria of exclusion included pregnancy, end stage renal failure, acute blood loss for any cause, chronic liver disease and terminal illness. Moreover, patients on supplements that contain multivitamins and vitamin D were also excluded. The authors informed the parents/guardians of patients about the study design, and the medicines used in this study. An informed consent was obtained from parents/guardians of patients before being included into the study.

Intervention

We designed an eight-week treatment protocol in which each patient with beta thalassemia major (50 patients) were administered orally 100,000 IU of vitamin D ampoule every two weeks, a total of four single doses were administered. The enrolled patients were advised to put the content of the ampoule into a half cup of milk and drink it. We performed patient follow-up monthly when they visited the center at the time for blood transfusions, in order to ensure patient safety and compliance. The patients/guardians of the patients were advised not to take any other supplements.

Biochemical and hematological tests

After 12 hours of overnight fasting, a blood sample was obtained by venipuncture from each patient before blood transfusion at baseline and after initiation of the eight-weeks commencement with vitamin D supplement. Part of the blood was drawn into ethylene diamine tetraacetic acid tubes and used for the determination of hematological indices using automated hematological analyzer, Coulter machine (Swelab alpha, Sweden). The remaining part of the blood was kept into plain tubes and allowed to clot before centrifuging at (3000 rpm, for 20 min) to obtain the serum for the determination of serum vitamin D (measured by Cobas e411), calcium, iron and ferritin levels. Serum IL-2, IL-10 and IL-6 were determined by using the technique of enzyme linked immunosorbent assay (ELISA) according to the manufacturer's instructions.

Statistical analysis

The results expressed as number, percentages and Mean ± SD. The data were analyzed by using two-tailed paired t-test taking the p-value of ≤ 0.05 as a lowest level of significance. All the data were analyzed using Microsoft Excel 2010 program for Windows (Microsoft Cooperation, Redmond, USA).

Results

The study included 46 beta thalassemia major patients with the mean age of 10.9±3.7 (6–18) years, 24 were males and 22 were females. Body mass index was 15.9±2.5. The descriptive statistics are shown in (Table 1). Vitamin D produced significant increase in serum vitamin D and calcium levels (p<0.001), the baseline vitamin D level improved from (15.70±7.52 ng/ml to 60.98±67.78 ng/ml) following supplementation (*P* < 0.001), and significant reduction in serum ferritin levels (p=0.046) (Table 2). Vitamin D3 supplements significantly decreased the pro-inflammatory cytokine interleukin-6 levels from (12.9±2.8 to 11.4±2.0, P<0.001), and elevated the serum levels of IL-2 and IL-10 (anti-inflammatory cytokine) from (11.55±1.96 to 12.39±2.15, P<0.001), and from (6.60±1.17 to 7.62±1.30, P<0.001) respectively (Table 3). Vitamin D supplementation produced a significant inverse correlation (r=0.258, p=0.015) with serum ferritin level (Figure 1). But there was no significant change in the hematological indices (Table 4).

Table 1: Characteristics of patients

Characteristics	**Results**
Sex (Male: Female)	24:22
Age (year)	10.9±3.7
Residency **Urban** **Rural**	 27 19
History of splenectomy	7
Body weight (kg) **Height (cm)** **Body mass index (kg/m^2)**	29.0±8.7 133.7±12.2 15.9±2.5

Notes: The results are expressed as number, and mean ± SD.

Table 2: Effect of vitamin D supplementation on the laboratory biochemical tests of thalassemia patients

Biochemical tests	**Before treatment (n=46)**	**After treatment (n=46)**	**p-value**
Serum vitamin D (ng/ml)	15.70±7.52	60.98±67.78	<0.001
Serum calcium (mg/dl)	8.74±0.41	9.30±0.5	<0.001
Serum ferritin (ng/ml)	1681.9±1273.3	1519.2±990.93	0.046

Notes: The results are expressed as mean± SD. P-value was calculated using two-tailed independent two sample t-test.

Table 3: Effect of vitamin D supplements on the inflammatory and cytokine markers

Inflammatory and cytokine markers	Before treatment	After treatment	P-value
Erythrocyte sedimentation rate (mm/h)	28.5±17.6	25.7±16.3	0.324
Serum Interleukin-2 (pg/ml)	11.6±2.0	12.4±2.2	<0.001
Serum inerleukin-6 (pg/ml)	12.9±2.8	11.4±2.0	<0.001
Serum interleukin-10 (pg/ml)	6.7±1.2	7.6±1.3	<0.001

Notes: The results are expressed as mean ± SD. P-value was calculated by using Chi-square test for category data and two-tailed paired t test for continuous data.

Table 4: Effect of vitamin D supplements on the red cell-related indices

Red cell-related indices	Before treatment	After treatment	P-value
Red cell count (x 10^6)	2.990±0.336	2.963±0.355	0.541
Hematocrit (%)	22.9±2.9	22.9±2.8	0.966
Hemoglobin (g/dl)	8.0±0.9	8.0±0.9	0.954
Mean corpuscular volume (fL)	76.7±4.5	77.5±4.3	0.086
Mean corpuscular hemoglobin (%)	26.9±1.6	27.1±1.7	0.129
Mean corpuscular hemoglobin concentration (g/dl)	35.0±1.0	35.0±0.9	0.962
Red distribution width (%)	17.3±5.1	17.5±5.5	0.689
Serum iron (µg/dl)	202.9±62.3	195.5±69.8	0.389

Notes: The results are expressed as mean ± SD. P-value was calculated by using two-tailed paired t test.

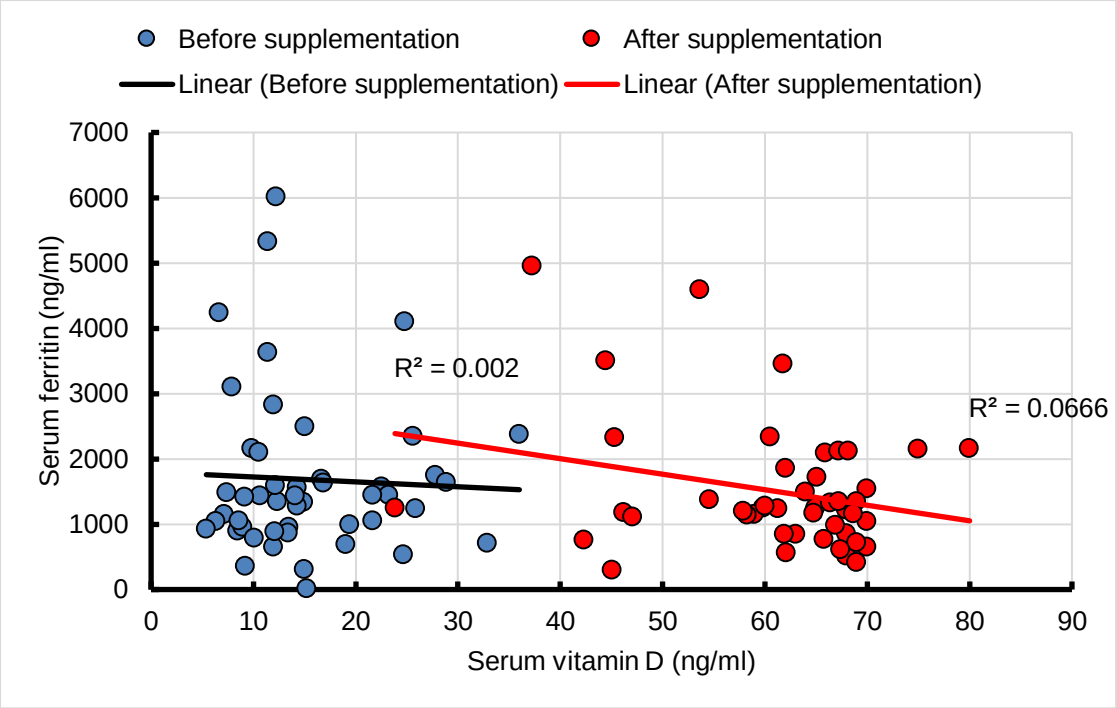

Figure 1. Effects of vitamin D supplementation on the non-parametric correlation between serum vitamin D with serum ferritin.

Vitamin D supplementation produced a significant inverse correlation (r=0.258, p=0.015) with serum ferritin level.

Discussion

This clinical study evaluated the clinical and biochemical outcomes after adjunct use of vitamin D with beta thalassemia major patients. The results of this open-label clinical study clearly demonstrated that vitamin D supplement for eight weeks significantly improved the ferritin status and inflammatory markers compared with before commencement of vitamin D supplement. This finding is a very interesting observation since there are many observational studies that support the anti-inflammatory role of vitamin D but we attempted to assess its effect on beta thalassemia patients [19,20]. Results of this study show that 8 weeks of vitamin D supplementation significantly increased the serum level of vitamin D and calcium levels. This finding is in agreement with previous studies that studied the efficacy of vitamin D3 in thalassemia, the majority of the patients showed an improvement in vitamin D level [21]. Vitamin D deficiency in thalassemia has been connected to malabsorption of vitamin D along with insufficient dietary consumption [22]. Another possible explanation is hepatic dysfunctions which result in defective hydroxylation of vitamin D resulting in reduced level [11]. Different authors also stated that vitamin D deficiency might be caused by hepatic iron-overload [23].

According to the present results, vitamin D produced a significant elevation in the serum levels of IL-2 and IL-10, additionally a significant reduction in the level of the pro-inflammatory cytokine IL-6, this points to the anti-inflammatory role of vitamin D in these patients. The anti-inflammatory role of IL-10 is well known; it can be viewed as the most important anti-inflammatory cytokine in human beings. Researchers have observed the effects of this cytokine as it limits secretion of proinflammatory cytokines, such as TNF-alpha, IL-1, IL-6, and IL-12, and by keeping proinflammatory events well controlled, it protects against excessive immune responses and tissue damage [24,25]. Numerous studies have confirmed the anti-inflammatory actions of vitamin D by

downregulating proinflammatory cytokines, while upregulating anti-inflammatory cytokines such as IL-10 [26]. Additionally, IL-2 controls inflammation and has the ability to induce the *in vitro* growth of activated T cells [27]. The results of another study demonstrated a weak negative association of serum vitamin D with IL-6 and leptin [28]. These results were consistent with a previous report that observed vitamin D deficiency was associated with more pro-inflammatory cytokines as compared with insufficiency or sufficiency status in elderly adults [19]. Moreover, the results of the present study reported that vitamin D did not alter the hematological indices, which indicates that vitamin D does not exert any effect on cell volume or morphology the blood cells in beta thalassemia patients. This finding is consistent with a study that observed deficiency of vitamin D had no effect on hematological indices in adolescents before versus after correction of serum vitamin D level [29]. Other studies observed that vitamin D has inhibitory impact on erythropoiesis by exhibiting inverse correlations between serum vitamin D and hemoglobin (Hb), red cell count and mean corpuscular hemoglobin in healthy teenagers by a mechanism is yet unidentified [30].

An interesting observation of this study was that the serum level of ferritin was significantly reduced post vitamin D supplementation when compared to the baseline. This finding can be explained by the effect of vitamin D on reducing hepcidin, which in turn reduces iron absorption from the enterocytes and hence the serum ferritin level declines. Hepcidin is known as the major iron-regulatory hormone [31,32]. It is primarily synthesized in the liver due to elevated plasma or tissue iron [33]. Hepcidin concentration is significantly higher in chronically transfused patients due to iron overload, such as in thalassemia [34]. Ferritin is an indicator of inflammation and a marker of iron store. In patients with anemia of chronic diseases and inflammation, vitamin D may decrease ferritin level for the improvement of inflammation status [35]. Hepcidin is also increased by inflammatory cytokines such as IL-6 [36]. Accordingly, the adjunct use of vitamin D in this study significantly reduced the pro inflammatory cytokine IL-6, which can also explain that the high level of hepcidin was attenuated by administering vitamin D. Since IL-6 can increase the hepcidin level, the down regulatory effect of vitamin D on the pro inflammatory cytokine IL-6 offers more benefit because as a result of reduction of IL-6, consequently hepcidin will be reduced [26]. Numerous studies have reported the inverse relationship between vitamin D and hepcidin [37-39]. Serum ferritin levels are modulated by hepcidin, which helps in reducing iron absorption from the intestine [40,41]. Serum hepcidin positively correlates with serum ferritin, reflecting the regulation of both proteins by iron stores [42].

Conclusion

We concluded that Vitamin D3 supplement improves the level of anti-inflammatory cytokines by enhancing anti-inflammatory and lowering pro-inflammatory cytokines in beta thalassemia major patients. Additionally, vitamin D supplementation may improve serum ferritin levels and may reduce the burdens of iron overload in thalassemia patients.

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Disclosure

The authors report no conflicts of interest in this work.

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